PRACTICAL PHYSIOLOGICAL CHEMISTRY.

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Practical Physiological Chemistry

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PREFACE TO THE FIRST EDITION.

My aim in writing this book has been to present to the student a series of exercises which can be successfully carried through in ordinary class work.

Too often a student is discouraged in his work and displeased with his Text-Book by finding that a minute care in following the instruction given fails to produce the specified result. I trust that no such difficulty will be met with in working through this Book. Each and every exercise given here I have first worked through and obtained the result stated. All I ask of the student is a zealous and interested care and he will then have no difficulty in performing the experiments and learning the lessons they teach.

The ground covered is more than is at present necessary for most examinations in medicine, but I feel that this is justified by the growing importance of the subject and the increasing standard of the knowledge of it required of candidates at these examinations.

A special feature of the book is the notes that follow certain of the exercises. These notes summarise a series of exercises, indicate the special precautions that are necessary for success or give the probable reasons for an apparent failure in the performance of a given exercise. They should be carefully studied both before and after the exercise to which they refer. At the end of the book spaces are provided for the student to draw various crystalline forms from preparations made by himself. I consider this a more instructive plan than giving illustrations of typical crystals, which often differ considerably from those prepared in class work. A blank chart for recording the absorption spectra of various pigment solutions and colour reactions is also added. The drawings should be shown to the demonstrator of the class for comments or corrections.

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November, 1904

PREFACE TO THE THIRD EDITION.

THE present volume is an outcome of the two editions of the Author's "Practical Exercises in Physiological Chemistry."

The increasing importance of the science to medical men has created a demand for a book that embodies precise instruction for class-work with an account of the properties and significance of the more important physiological substances. The present work is an attempt to realise these desiderata.

The Author wishes to draw particular attention to the analytical methods. It is lamentable that for the investigation of the nitrogenous excretion of a patient, the average medical man has at present only one method at his command. That method, the hypobromite, is notoriously unreliable, and the conclusions drawn from it may be extremely misleading. It is sincerely hoped that all medical students will be taught the microchemical methods of urinary analysis introduced by Folin. The Author is convinced that they are reliable, and that the average medical man could conduct them rapidly with a very small amount of special apparatus. If such training were universally adopted in England, an enormous amount of clinical material that is now wasted would become available for research, and a rapid increase in our knowledge of physiology and pathology would inevitably follow.

The qualitative methods for urinary analysis also have been considerably modified in recent years, especially in regard to

sugar. Fehling's method, that has for so long been the crucial test, is unreliable. It should be supplanted as soon as possible by more conclusive methods, such as those described in the section on glucose in urine.

By a judicious selection of exercises the book can be adapted for elementary or advanced classes.

The Author gratefully acknowledges his indebtedness to Mr. H. M. Spiers, of Caius College, for invaluable help in reading the proofs, and to Messrs. J. Griffin & Sons and Messrs. Baird & Tatlock for the loan of certain of the diagrams.

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LIST OF ILLUSTRATIONS.

ALTERATIONS, CORRECTIONS AND OMISSIONS.

The reader is advised to make the necessary corrections without delay

p. 33, l. 2. *For* "lactore" *read* "lactone."

p. 46, l. 16. *For* "Achrodextrin" *read* "Achroodextrin."

p. 153, Ex. 273. *For* "To the yellow" *read* "Heat the yellow." Insert a comma after "exercise."

p. 162, Ex. 293. *For* "5 or 6" *read* "10."

p. 164, Ex. 298. Bial's reagent consists of 1 to 1·5 gm. orcine, 500 c.c. of concentrated hydrochloric acid and 30 drops of a 1 p.c solution of ferric chloride.

p. 174, l. 6 from bottom. *For* "35" *read* "50."

p. 177, l. 10. *For* "3" *read* "4."

p. 177, l. 21 to 23. *Delete* "To each flask . . . Nessler's solution."

p. 177, l. 3 from bottom. *For* "20" *read* "10."

p. 184, l. 13. *For* "179" *read* "177."

p. 185, l. 3. *For* "0·45" *read* "0·045."

p. 190. Preparation of a stable solution of uric acid. Dissolve 1 gm. of uric acid in 200 c.c. of 0·4 p.c. lithium carbonate. Add 40 c.c. of 40 p.c. formaldehyde. Shake and allow to stand a few minutes. Add 20 c.c. of normal acetic acid. Make up to 1 litre with water. Standardize colorimetrically the next day against a freshly prepared solution of uric acid (*see* p. 190), using 5 c.c. of the formalin-uric-acid solution. This should contain very nearly 1 mg. of uric acid that reacts with Folin's reagent. The solution is quite stable.

p. 198, Ex. 322. Benedict's sulphur reagent is -

Crystallised copper nitrate, 200 gm.
Potassium chlorate, 50 gm.
Distilled water to 1 litre.

p. 205, l. 11 from bottom. *For* "249" *read* "248."

CHAPTER I.

THE PROTEINS.

These bodies are composed of certain amino-acids and bases condensed in varying proportions.

A. Classification.

1. **Protamines.** Basic substances, containing a high percentage of nitrogen and formed almost entirely of bases. They are found in ripe spermatozoa and ova. They form salts with acids.

2. **Histones.** Similar to the protamines, but less rich in nitrogen and bases. Found in unripe spermatozoa, the stroma of red corpuscles, and in the thymus. They are precipitated by ammonia.

3. **Globulins** insoluble in water
4. **Albumins** soluble in water } coagulated by boiling.

5. **Glutelins.** Insoluble in water and alcohol; soluble in dilute acid or alkali
6. **Gliadins.** Insoluble in water: soluble in 75 % alcohol } Found in cereals.

7. **Sclero-proteins.** Forming the skeletal structure of animals; *e.g.* keratin, elastin, collagen (the anhydride of gelatin).

8. **Phospho-proteins.** Proteins rich in phosphorus, *e.g.* caseinogen of milk and vitellin of egg-yolk.

9. **Conjugated-proteins.** Proteins joined to a non-protein ("prosthetic") group.

(i) *Chromoproteins.* Protein + pigment molecule, *e.g.* haemoglobin.

(ii) *Nucleoproteins.* Protein + nuclein or nucleic acid.

(iii) *Glucoproteins.* Protein + carbohydrate, *e.g.* mucin.

Hydrolysed Proteins. [illegible]

Metaproteins.

Albumoses or Proteoses.

Peptones.

Polypeptides formed of [illegible].

B. General Properties.

[illegible] are dependent on the [illegible] constituent radicles. (See I., II.)

They are precipitated by the so-called alkaloidal reagents [illegible].

They are precipitated by [illegible] metals [illegible].

They are colloidal. That is, they do not diffuse through animal membranes, and the large molecules tend to aggregate together under the influence of heat, neutral salts, etc., to form a precipitate or coagulum.

Solubilities of the chief proteins.

S Soluble. I Insoluble.

	Water	Dilute Acids	Dilute Alkalies	Alcohol	Absolute Alcohol	Ether
Globulin	I	S	S	S	I	I
Albumin	S	S	S	S	S	I
Metaprotein	I	I	S	S	I	I
Primary Albumose	S	S	S	S	I	I
Secondary Albumose	S	S	S	S	S	I
Peptone	S	S	S	S	S	S
Caseinogen	I	I	I	S	I	I
Nucleoprotein	I	I	I	S	I	I
Mucin	I	I	I	S	I	I
Gelatin	S*	S*	S*	S*	I	I
Keratin	I	I	I	I	I	I

* If warmed

C. The Colour Reactions of Proteins.

[illegible]

1. **The Xanthoproteic reaction.** To 5 c.c. of the protein solution in a test tube add about one-third of its volume of strong nitric acid. A white precipitate is formed. Boil the mixture. The precipitate turns yellow and partly dissolves to give a yellow solution. Cool under the tap and add strong ammonia till the reaction is alkaline. The yellow colour is turned to orange.

Notes. 1. The essential feature of the reaction is the yellow colour which develops when the solution is boiled with strong nitric acid, and that this yellow colour is intensified by the addition of excess of ammonia.

2. The precipitate is due to the formation of a precipitate by the nitric acid and a dilute solution of albumin is coagulated by being boiled with strong nitric acid. (See Ex. 7.) But the xanthoproteic reaction is due to the formation of a soluble nitro-derivative of the protein and not to the precipitate which is formed.

3. The yellow colour is due to the formation of a nitro-compound of the aromatic substances, e.g. tyrosine, tryptophane, and phenylalanine.

4. The aromatic substances in the protein molecule that are responsible for the reaction are tyrosine, tryptophane, and phenylalanine.

5. Other coloured substances, such as indigo, do not give the xanthoproteic reaction.

2. **Millon's reaction.** Treat 5 c.c. of the protein solution with half its volume of Millon's reagent. A white precipitate is formed. Boil the mixture. The precipitate turns brick-red in colour, or disappears and leaves a red solution.

Notes. 1. The essential feature of the reaction is the red colour on boiling. The white precipitate in the cold is due to the action of the mercuric nitrate on the proteins. (See Ex. 7.)

2. A white precipitate is also obtained with solutions of urea. (See Ex. 12.)

3. Sulphates give a white precipitate of mercurous sulphate.

4. The reagent is made by dissolving 30 c.c. of mercury in 570 c.c. of concentrated nitric acid and diluting with twice its bulk of water. It contains mercurous and mercuric nitrates, excess of nitric acid, and a small amount of nitrous acid.

5. The reaction should never be attempted with a strongly alkaline fluid, since the alkali will precipitate the yellow or black oxides of mercury.

The glyoxylic reaction [illegible] Treat [illegible] the fluid with the same bulk of "reduced" acid. Mix and add an equal volume of concentrated sulphuric acid, [illegible] down the side of the tube. A purple tint at the [illegible] of the fluids. Mix the fluids by taking the tube gently from side to side. The purple colour spreads through the whole fluid.

[illegible]

A [illegible] add to [illegible] Warm all the [illegible] Cool and add [illegible] [illegible] Boil. [illegible]

B [illegible] with distilled water [illegible] and heat [illegible] for the [illegible] Filter off the insoluble [illegible] oxidate, wash with [illegible] water and [illegible] above.

[illegible]

[illegible]

[illegible]

[illegible]

4. **Piotrowski's reaction (the biuret reaction).**

. **The sulphur reaction.**

. **Molisch's reaction.**

D. The Albumins and Globulins of Blood Serum.

7. Take the specific gravity by floating a clean, dry urinometer in a cylinder containing the serum, and noting the graduation where the stem of the urinometer is level with the surface of the fluid. It is usually about 1030 (water being taken as 1000).

8. Take the reaction of the serum to litmus paper. It is alkaline.

Heat-coagulation of albumins and globulins.

When a solution of albumin or globulin is heated under certain conditions, the protein separates from

solution in a form that is insoluble in water, salt solutions, dilute acids and alkalies. This change is known as "heat-coagulation."

It seems to consist of two processes:—

1) The interaction of protein and water ("denaturation").

2) The subsequent agglutination and separation of the product.

The first process may take place without the second.

Both processes are much affected by the reaction of the solution and by the presence of neutral salts.

In general it might be stated that an increase in acidity or alkalinity up to a certain point favours denaturation but decreases the tendency to agglutination. The reverse is true for neutral salts.

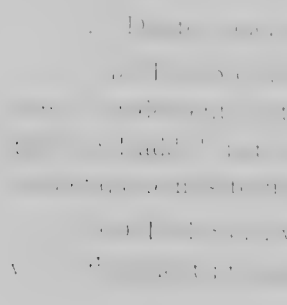
The best medium for obtaining heat-coagulation is one very slightly acid and containing a small amount of a neutral salt, preferably that of one of the alkaline earths, *e.g.* calcium chloride.

The material produced by heating the protein with water can be regarded as a hydrolytic product, metaprotein. If there be a sufficient amount of acid or alkali present there is no agglutination of this unless a certain amount of neutral salt be present. In general it can be stated that the smaller the amount of neutral salt present, the smaller is the amount of alkali or acid necessary to inhibit agglutination.

When a protein is treated with a dilute acid, *e.g.* HCl, a salt is formed. This is hydrolysed by water into protein and free HCl, which can be completely removed by prolonged dialysis. But if such a solution of protein in weak acid be boiled, the coagulum that forms consists of the salt, that is, the HCl is partly removed from the solution on coagulation.

As regards the condition of the protein in "solution," it has been shewn that the particles are really suspended in the "solvent" and that they carry an electrical charge. This charge determines the stability of the system, and any factor tending to reduce the charge promotes precipitation or coagulation. The sign of the charge on the particle is determined by the chemical nature of the particle, and may also depend on the nature of the solvent. Hardy has shewn that in the case of the proteins, which have amphoteric characters, the sign of the particle is positive when the fluid is acid and negative when the fluid is alkaline. When a salt is added to such a colloidal solution it exerts a coagulative effect which depends upon one of its ions, the coagulating ion being that which carries a charge opposite in sign to that of the particle. The coagulative power increases rapidly with the valency. Thus in acid solution the protein has a positive charge and so is precipitated by negative ions, and it is found that the potassium salt of citric acid (trivalent) is much more effective than the potassium salt of sulphuric acid (divalent), and this more than the potassium salt of hydrochloric acid. In alkaline solutions on the other hand the cation is the coagulative ion and cerium chloride ($CeCl_3$) is more efficient than barium chloride ($BaCl_2$) and this more than sodium chloride ($NaCl$).

In the following two exercises, the explanations offered in the notes are sufficient for elementary students



a three drops of strong nitric acid. The precipitate does not dissolve.

(c) Treat 5 c.c. of serum with 4 per cent. hydrochloric acid, drop by drop, till the solution is clear (about 5 drops are usually necessary). Boil. A precipitate is not formed. Cool the tube and add 2 per cent. sodium carbonate, drop by drop. A precipitate is formed, which is dissolved in excess.

(d) Boil 5 c.c. with two drops of 4 per cent. Na_2CO_3. A precipitate is not formed. Cool the tube and add 4 per cent. acetic acid. A precipitate of globulin is formed, insoluble in excess of acid.

[illegible]

[illegible]

[illegible]

[illegible]

12. [illegible] in ... [illegible] ... [illegible]

[illegible] ... Shake well ... [illegible] ... the precipitate with water. [illegible]

[illegible]

13. To about 5 c.c. of diluted serum add a few drops of strong acetic acid. A precipitate is not formed. [illegible] of the ... [illegible] ... precipitate is formed.

Note. [illegible]

Precipitation by Alkaloidal Reagents.

14. Treat 5 c.c. of diluted serum with two or three drops of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed. Boil. The precipitate does not dissolve.

Note. [illegible] (See [illegible].)

[illegible]

15. To some serum diluted about ten times and acidified with dilute [illegible] add a few drops of [illegible]. A white or [illegible] precipitate is formed.

16. Treat 5 c.c. of diluted serum with an equal volume of Esbach's solution. A yellowish precipitate is formed.

[illegible]

17. Acidify some diluted serum with dilute hydrochloric acid and add a few drops of potassio-mercuric iodide (Brucke's reagent). A white precipitate is formed.

[illegible]

18. Acidify 5 c.c. of serum with dilute hydrochloric acid and add a solution of phosphotungstic acid. A white precipitate is produced.

Precipitation by the salts of the Heavy Metals.

19. To diluted serum add a few drops of mercuric nitrate solution. A white precipitate is formed, soluble in saturated sodium chloride solution, and reprecipitated from this by the addition of dilute hydrochloric acid.

20. To diluted serum add ferric chloride solution. A precipitate is formed soluble in excess.

21. To diluted serum add copper sulphate solution drop by drop. A bluish-grey precipitate is formed.

22. To diluted serum add a solution of lead acetate or basic lead acetate. A white precipitate is formed.

The remaining exercises of this section deal with the special physical properties of the globulins and albumins of serum.

Globulins are insoluble in distilled water, but soluble in dilute acids and alkalies, and in weak solutions of neutral salts.

A neutral solution in a dilute salt is coagulated on boiling.

A solution in dilute acid or alkali is converted into a solution of metaprotein on boiling.

If the globulin be dissolved in a minimum amount of a neutral salt solution and the solution be diluted with

several volumes of distilled water, the globulin is partially precipitated, for a certain *concentration* of salt is necessary to keep the globulin in solution. If the globulin be dissolved in dilute acid or alkali, there is no precipitation on dilution.

The globulins are completely precipitated by full saturation with magnesium sulphate or by half-saturation with ammonium sulphate, *i.e.* by treatment of the solution with an equal volume of a saturated solution of ammonium sulphate.

Albumins are soluble in distilled water, dilute salt solutions, dilute acids and alkalies.

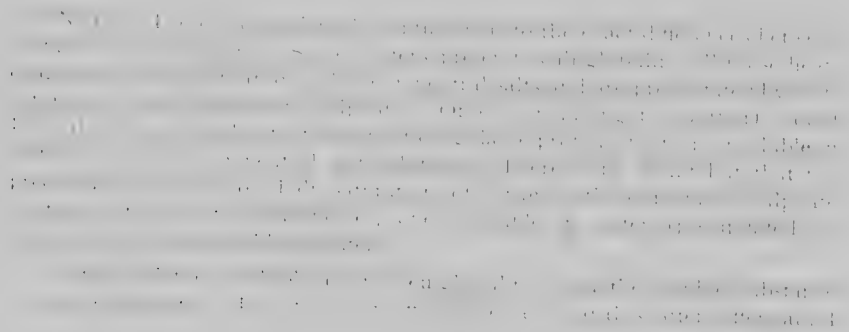
A neutral solution in water or salt is coagulated on boiling.

A solution in dilute acid or alkali is converted to a solution of metaprotein on boiling.

Solutions of albumins are only partially precipitated by saturation with magnesium sulphate or by half-saturation with ammonium sulphate if the reaction of the solution be neutral or alkaline.

They are more completely precipitated by solutions of these substances in the presence of acid.

They are completely precipitated by full saturation with ammonium sulphate from a neutral, acid, or alkaline solution.



from the albumin that is only precipitated by full saturation with ammonium sulphate. Therefore it seems better at present to restrict the term globulin to that portion of the serum proteid that is insoluble in water.

23. Dilute 5 c.c. of serum with 50 c.c. of distilled water. A faint cloud of serum-globulin is formed. Add 4 p.c. hydrochloric or 1 p.c. acetic acid, drop by drop. The cloud becomes denser and then clears up.

NOTE.—The globulin in the separated clot can be extracted by means of dilute alkalies. Dilution alone precipitates it. The solution can be now treated with just enough acid to neutralise the alkali. The larger fraction of the globulin is then precipitated. When acid is added the globulin dissolves.

24. Prepare a suspension of globulin by the following method. To 15 c.c. of serum in a beaker add 2 c.c. (about 30 drops) of 1 p.c. acetic acid and 150 c.c. distilled water. Stir and allow the mixture to stand for about 20 minutes. A precipitate of globulin settles down. Very carefully pour off the supernatant fluid and divide the suspended globulin into two equal portions in clean test-tubes. With these perform the two following exercises.

25. Add a 5 p.c. solution of sodium chloride, drop by drop, till the globulin has just dissolved. Divide the solution into three portions, A, B and C.

(*a*) Boil. The protein is coagulated.

(*b*) Dilute with about five volumes of distilled water. The globulin is partially reprecipitated.

(*c*) Treat with an equal volume of saturated ammonium sulphate solution. The globulin is reprecipitated.

26. Add 4 p.c. HCl, drop by drop, till the globulin has just dissolved. Divide the solution into three portions, D, E and F.

(*d*) Add 2 p.c. sodium carbonate solution till the globulin is partially reprecipitated. One or two drops only are necessary. Now add a few drops of 5 p.c. sodium chloride. The precipitate of globulin redissolves.

(*e*) Boil the solution. The protein is not coagulated. Cool under the tap and add 2 or 3 p.c. sodium carbonate

precipitate is the metaprotein that has been formed by boiling. Now add a few drops of very dilute solution of hydrochloric acid. The precipitate of metaprotein does not dissolve.

(c) Dilute with about twice its volume of distilled water. The globulin is at once thrown out of solution.

3. Mix about 10 c.c. of undiluted serum with an exactly equivalent quantity of a saturated solution of ammonium sulphate. A thick white precipitate is formed consisting of the whole of the globulin and a portion of the albumin. Filter through a dry filter paper into a dry test-tube. Label the filtrate A. Scrape the precipitate off the paper and treat it with distilled water. The precipitate dissolves, the ammonium sulphate adhering to it forming a dilute salt solution which allows the globulin to go into solution. Boil a portion of this solution. A heat-coagulum is formed.

4. Filtrate A contains serum-albumin in the presence of half-saturated ammonium sulphate. Apply the following tests:

(a) Boil a portion. A heat-coagulum is formed.

(b) To another add one drop of strong acetic acid. A white precipitate of serum-albumin is formed.

(c) Grind the remainder in a mortar with solid $(NH_4)_2SO_4$ till the fluid is saturated. A white precipitate of serum-albumin is formed. Filter off the precipitate and test the filtrate for proteins either by boiling or by the glyoxylic or xanthoprotein reactions. Proteins are absent, showing that all the proteins of serum are precipitated by complete saturation with $(NH_4)_2SO_4$.

Note. A certain number of drops of a solution of sodium acetate must be added to the ammonium sulphate to prevent the precipitate that would be present if boiled from dissolving in the filtrate. A heat-coagulum is then formed.

5. Serum has been dialysed in parchment tubes for two or three days against repeated changes of distilled water. Note the heavy precipitate of serum-globulin that has fallen to the bottom of the tube.

(c). Dilute 5 c.c. of serum with five times its volume of tap water, add a drop or two of 2 per cent. calcium chloride and a drop or two of neutral litmus. Boil the mixture over a [illegible] whilst boiling cautiously add 1 per cent. acetic acid till the reaction is *faintly* acid. Filter, and test the filtrate for proteins by the usual colour tests. If the operation has been carried out successfully the filtrate will be found to be nearly free from proteins.

Note. [illegible] globulins from solution. [illegible] (See Chapter I., II.)

It is most important that the acid [illegible] added in very small excess. The small amount of calcium chloride added [illegible]

E. The Chemistry of Egg-white.

1. In egg white which has been well beaten with a whisk (to break up the containing membranes), and diluted with four times its volume of distilled water, note a precipitate of ovo-mucin and globulin. Perform the following tests:

(*a*) Take the reaction to litmus. It is alkaline.

(*b*) Cautiously neutralise with dilute acetic acid. A slight increase in the precipitate of ovo-mucin and globulin is noticed. Remove this by filtration if necessary, and with the filtrate perform the following reactions:

(*c*) Boil a portion. A coagulum is formed, indicating the presence of either a globulin or an albumin.

(*d*) Make another portion very faintly alkaline by the addition of a drop or two of 2 per cent. Na_2CO_3. Now add an equal bulk of saturated $(NH_4)_2SO_4$. A slight precipitate of globulin or albumin is formed. Filter this off, and boil a portion

of the filtrate with a dropped 1 per cent. acetic acid. Vegetable albumose is formed. Saturate the remainder of the filtrate with $(NH_4)_2SO_4$ by grinding with the solid in a mortar. A precipitate of albumose is formed.

Compare in a similar manner egg-albumin by heating. Filter and apply Millon's reagent to the white precipitate. Proteose is found in small amount. Deuteroalbumose is formed. It is precipitated by dilute acetic acid. It is precipitated by saturation with $(NH_4)_2SO_4$, and also by strong alcohol.

The crystallisation of egg-albumin. Separate the white from a number of new-laid eggs, take care not to allow any of the yolk to mix with the white. Measure the white and add to it an exactly equal volume of a neutral dilute ammonium sulphate solution, which should be prepared in portions and mixed immediately after every addition. Neutralise the mixture with ammonia and filter. Filter the mixture through a large plaited filter paper. Measure the filtrate. Take 100 c.c. of it and cautiously treat it with 10 per cent. acetic acid from a burette, noting the original level of the acid in the burette. Add the acid a drop or two at a time, shaking gently the whole time, until the precipitate produced at each addition no longer disappears on shaking, and the whole mixture is rather opalescent. The process is a slow one, and care has to be taken not to add too much acid at once. The globulin will be precipitated in the finely divided condition, and will not be suspended in the fluid as a gelatinous precipitate. When you have arrived at the permanent precipitate for 100 c.c. of the fluid, note the level of the acid in the burette, and add to the remainder of the filtrate the amount already added. A coarse precipitate is then produced. Note the amount of acid that has been used for 100 c.c. and treat the remainder of the filtrate with a corresponding amount of acid. Mix the two portions thoroughly and leave at the ordinary temperature. Note that the precipitate has increased somewhat in amount. Measure a drop of the suspension

F. The Gluco-proteins.

These bodies are conjugated proteins, the protein being united to a carbohydrate group.

They consist of the mucins and mucinoids or mucoids. The mucins are found in connective tissue and are secreted by certain of the salivary glands and various parts of the alimentary canal, notably the large intestine. Their solutions are viscous. They are soluble in dilute alkalies and are precipitated from solution by acetic acid the precipitate being insoluble in excess of acetic acid. They are also soluble in 0·4 per cent. hydrochloric acid. On hydrolysis with acids the sugar group is split off and will reduce Fehling's solution.

The mucoids are not so viscous and not so readily precipitated by acetic acid, the precipitate being soluble in excess. They are found in ovarian cysts and in white of egg (See Ex. 31 a).

Preparation of Mucin. Mince the submaxillary gland of an ox, grind with sand and add 1 per cent. NaOH (1 litre to 50 grams of the moist gland). Shake well in a large bottle from time to time and leave for about half an hour. Strain through muslin and filter through coarse filter-paper. (This crude solution should not be prepared too long before use, as mucin loses its characteristic properties if left standing with alkalies.)

(2) Add acetic acid drop by drop. A stringy precipitate is formed, insoluble in excess of the acid.

(3) Remove the precipitate on a glass rod, wash with water, and apply the usual colour reactions for proteins, *e.g.* xanthoproteic, glyoxylic, and Millon's. They are all given by mucin.

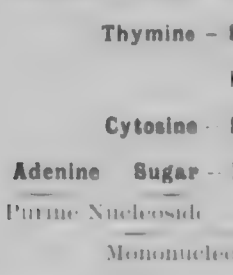
(4) Treat some of the precipitate with 1 per cent. HCl. The mucin dissolves.

(5) Treat some of the precipitate with 2 per cent. Na_2CO_3. The mucin dissolves.

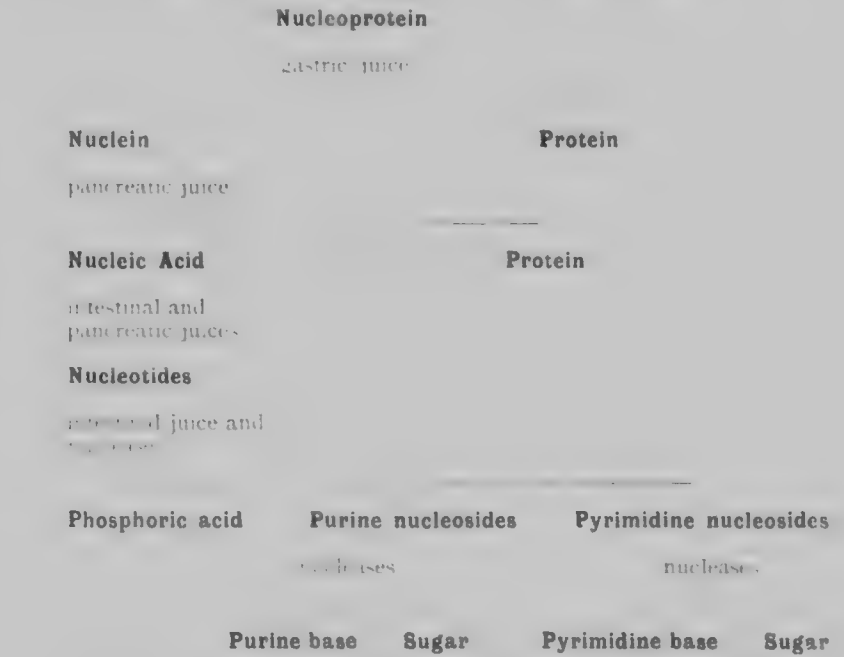
G. The Nucleoproteins and Nucleohistones.

These substances are conjugated proteins, the protein being in combination with nuclein. Nuclein is a protein combined with nucleic acid, a complex body rich in phosphorus. The nucleoproteins and nucleohistones are found in most tissues of the body, notably in those rich in cells, as the thymus, lymphatic glands, testes, pancreas, etc. They differ in the nature of the protein combined with nuclein. In the nucleoproteins it is of the nature of a peptone: in nucleohistone it is a histone. (See page 1.) The nucleic acids are polynucleotides, formed by the condensation of a certain number of *nucleotides*, which have the composition of a simple nucleic acid. The mononucleotides consist of phosphoric acid in combination with a *nucleoside*, a compound formed by the union of a sugar with a purine or a pyrimidine group. In many cases the sugar is a pentose (*d*-ribose), but in others it is a hexose which has not yet been identified.

The composition of the nucleic acid obtained from the thymus can be represented as follows:



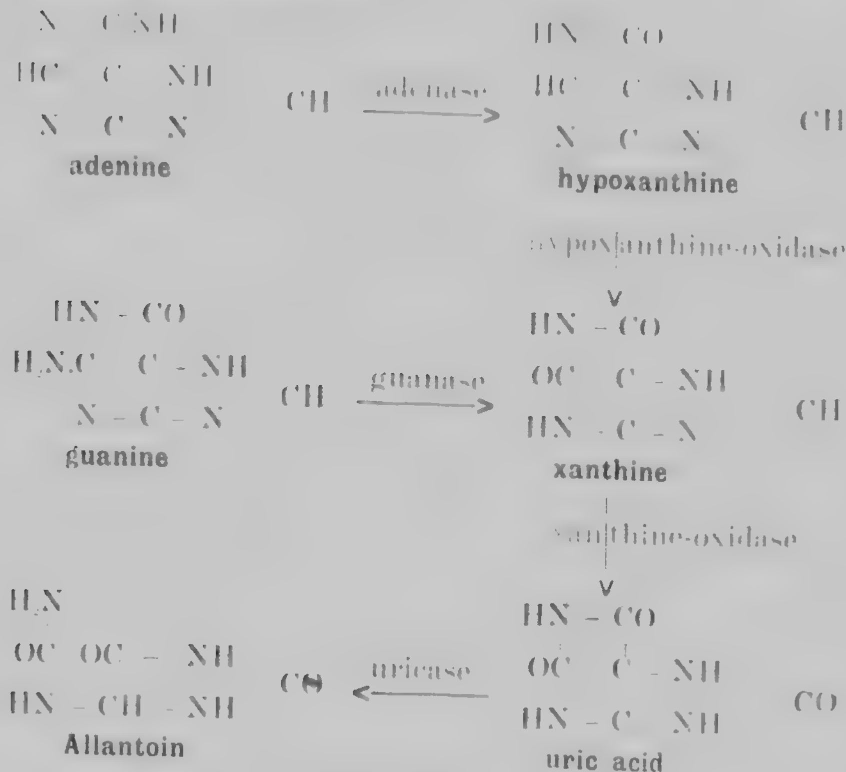
The hydrolysis of nucleoproteins is effected by gastric, pancreatic and intestinal juices, and by certain ferments, known as nucleases, found in the tissues. The action of these is shewn in the following scheme:



The purine bases found are guanine and adenine, which are converted by tissue ferments called guanase and adenase to xanthine and hypoxanthine respectively.

[illegible] xanthine [illegible] allantoin [illegible]

These reactions are of considerable importance in connection with the problem of the [illegible] acid excreted by the mammal.



Preparation. Lymphatic glands of the [illegible] are freed from fat, finely minced [illegible] [illegible] distilled water [illegible] toluol or chloroform [illegible] to prevent [illegible] [illegible] [illegible] stone.

Physical Properties. Nucleoproteins are acidic bodies which dissolve in dilute alkalies. The salt-like bodies thus formed are precipitated as the free acid by addition of dilute acetic acid. They dissolve to an opalescent solution in excess of strong acetic acid (distinction from mucins). Nucleohistone is precipitated as a calcium compound by 2 per cent. calcium chloride solution. Solutions are precipitated by half saturation with [illegible] sulphate.

treat the filtrate with about one-tenth its volume of strong nitric acid and one-third its volume of ammonium molybdate. Boil for two minutes. The yellow crystalline precipitate separating out in the side of the tube shows that such proteins are to be distinguished as contain phosphorus, that has been oxidised to phosphate by the fusing.

H. The Metaproteins.

The metaproteins are derived from the albumins and globulins by hydrolysis. This can be effected rapidly by dilute acids and alkalies at temperatures over 60° C. (see notes to Ex. 9): more slowly at body temperature. They are formed immediately by the action of strong mineral acids at room temperature. See Exs. 1 and 13.) They are insoluble in water, *strong* mineral acids, and all solutions of neutral salts, but are soluble in dilute acids or alkalies in the absence of any large amount of neutral salts. They are not thrown out of solution (in acid or alkali) by boiling. But if such a solution be neutralised or precipitated by the addition of an excess of a neutral salt, the suspended metaprotein is coagulated on boiling, so that it will no longer dissolve in acid or alkali.

Preparation. Egg white or serum is diluted with ten times its volume of either ·4 per cent. hydrochloric acid or 1 per cent. sodium hydrate and the mixture placed in a water bath or incubator at 40° C. for about twenty-four hours. The albumins and globulins are hydrolysed to metaprotein.

40. To about twenty-five c.c. add a few drops of litmus and carefully neutralise with 2 p.c. Na_2CO_3 or ·4 p.c. HCl. A bulky precipitate of metaprotein separates out. Filter. Scrape the precipitate off the paper and suspend it in a test-tube about half-full of water. Divide the suspension into six equal portions and with them perform the following six exercises:

11. Add some ·4 p.c. HCl. The precipitate dissolves. Neutralise with Na_2CO_3: the precipitate reappears.

12. Add concentrated HCl drop by drop. The precipitate dissolves with the first drop, and reappears when an excess is added.

13. Dissolve in a little ·4 p.c. HCl. Boil the solution: a coagulum is not formed. Cool under the tap and neutralise with p.c. Na_2CO_3. A precipitate is formed which is soluble in an excess.

14. Boil. Cool and add some ·4 p.c. HCl. The precipitate does not dissolve, *i.e.* metaprotein is coagulated when boiled in suspension.

15. Add a saturated solution of ammonium sulphate drop by drop. The precipitate does not dissolve in any dilution of the salt.

16. Dissolve in a little ·4 p.c. HCl. Treat the solution with an equal volume of saturated ammonium sulphate solution. The protein is precipitated.

I. The Albumoses and Peptones.

These hydrolysed proteins are obtained by the further action of acids or alkalies on globulins, albumins and metaproteins. They are best formed by the action of pepsin and hydrochloric acid on these proteins. Peptone is the end product of gastric digestion.

They are prepared on a commercial scale and sold as

(i.) Witte's peptone, which is prepared from fibrin and consists of a mixture of albumoses and peptone.

(ii.) Savory and Moore's peptone, which is prepared from meat, and only contains traces of albumoses.

The following scheme indicates the successive steps

in the digestion of fibrin by pepsin and 0·2 per cent hydrochloric acid

Fibrin

Soluble Globulin

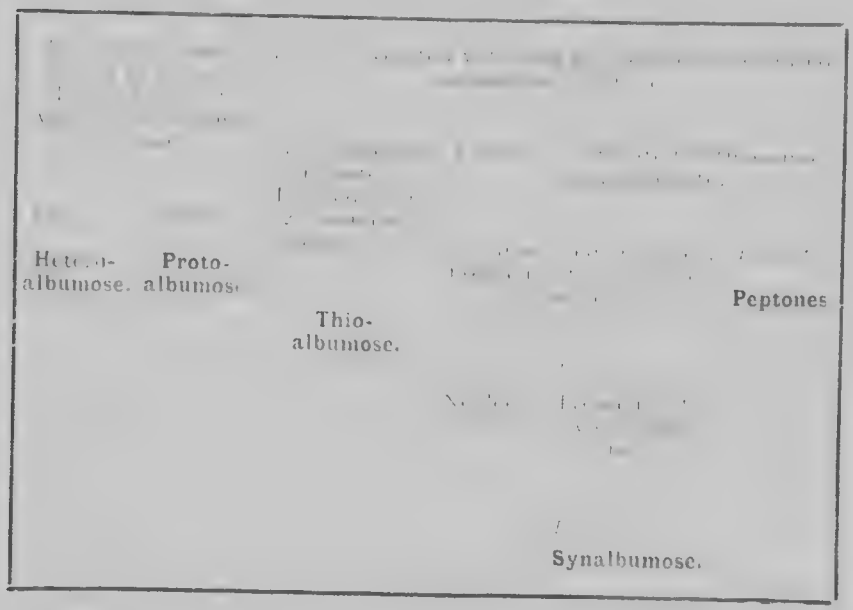
Metaprotein

Primary albumoses — Hetero-albumose. — **Secondary albumoses** — Thioalbumose

Peptones.

The following scheme shews the method adopted for the separation of certain of the albumoses.

Neutral Witte's peptone, treated with equal volume of saturated ammonium sulphate solution.



The **primary albumoses** are soluble in water, dilute acid, alkalies and salt solutions. Their solutions are not coagulated on heating. They are precipitated by half

saturation with ammonium sulphate. They give a precipitate that disappears on warming and reappears on cooling; react with nitric acid or potassium ferrocyanide and acetic acid. They also give a precipitate in the cold with copper sulphate.

They give all the ordinary proteid colour reactions with the exception of Molisch's.

The **secondary albumoses** have somewhat similar properties to those of the primary albumoses; but they are not precipitated by nitric acid, hydro-ferrocyanic acid or copper sulphate.

They require more than half-saturation with ammonium sulphate to precipitate them, but are completely precipitated by full saturation. Thio-albumose gives all the protein colour reactions and is particularly rich in sulphur hence its name.

Synalbumose gives the proteid reactions, with the exception of the glyoxylic test.

The **peptones** are very soluble proteids, of a low molecular weight, so that they slowly diffuse through parchment membranes. They are the only proteins not precipitated by full saturation with ammonium sulphate. They fail to give precipitates with Esbach's or Brücke's reagents or hydro-ferrocyanic acid, but are precipitated by other protein precipitants, as tannic acid, phospho-tungstic acid and lead acetate.

For the following reactions make a 5 per cent. solution of "Witte's peptone" in water. [illegible]

48. Boil the solution with a trace of acetic acid: it does not form a coagulum.

49. Add a little tannic acid: no precipitate is formed.

50. Add a little Esbach's or Brücke's reagent: a yellow or white precipitate is formed.

51. Add a little lead acetate solution: a white precipitate is formed.

52. To 2 c.c. of the 5 per cent. solution in a small beaker add 10 c.c. of a saturated solution of ammonium sulphate. A white precipitate of the primary albumoses is formed. Stir the mixture vigorously for a short time with a glass rod that has one end covered with a small piece of rubber tubing: allow to stand for a few minutes. The precipitate will usually gather together and can be almost completely collected as a gummy mass on the end of the rod. Transfer it to about 5 c.c. of hot water. The precipitate dissolves. Cool the solution and divide it into three portions.

(a) Add a drop of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed, which disappears on heating and reappears on cooling.

(b) To another portion add a few drops of strong nitric acid. A white precipitate is formed, which disappears on heating and reappears on cooling.

(c) To the third portion add a drop of copper sulphate solution. A white precipitate is formed.

53. The fluid from which the main mass of primary albumoses has been removed is filtered and treated in a beaker with a single drop of sulphuric acid, and then with ammonium sulphate that has been finely powdered in a mortar. The mixture is stirred vigorously till the fluid is saturated with the salt. A flocculent precipitate of the secondary albumoses (deutero-albumoses) is formed. Collect this on the rod as before, dissolve in a little water, divide the

solution into three parts, and repeat the three tests already performed with the primary albumoses. A precipitate is not formed by any of the reagents.

54. The fluid from which the secondary albumoses have been removed contains peptone. Filter it, and treat a portion of the filtrate with twice its volume of 40 per cent. sodium hydroxide and a drop of 1 per cent. copper sulphate. A pink colour appears, due to the presence of peptone.

Important Note.—This large excess of strong NaOH **must** be added in order to decompose the $(NH_4)_2SO_4$ with which the solution is saturated. The characteristic rose colour is only obtained when the alkalinity is due to NaOH, ammonia being quite inefficient.

1 saturated $(NH_4)_2SO_4$ solution contains about 3·75 grms. of the salt. This requires 2·27 grms. of NaOH. 10 c.c. of 40% NaOH, containing 4 grms. NaOH, is thus sufficient.

55. Evaporate a small portion of the original fluid to complete dryness, finishing the process on a water bath in order to prevent charring. Rub up the residue with successive small quantities of strong alcohol (95 per cent.). Add the extracts together, filter and evaporate them to dryness on a water bath. Dissolve the residue from this evaporation in a little water and test for proteins by the various colour tests. Only insignificant traces are present, showing that albumoses and peptones are insoluble in strong alcohol.

NOTE.—It is frequently desirable to remove all proteins from a solution before testing for certain substances, e.g. sugars, bile-salts, urea, etc. In the case of albumoses and peptones this can only be effected by the method described above, advantage being taken of the solubility of sugars, etc., in alcohol, and the insolubility of all proteins in the same. The aqueous solution prepared in this way will be spoken of as "an alcoholic extract."

Peptones. Use a 2 per cent. solution of Savory and Moore's peptone, which is usually free from albumoses.

56. Apply the usual colour reactions for proteins. They are all obtained.

NOTE.—The glyoxylic reaction may not be very intense, owing to the presence of chlorides in the preparation. Pure peptone, when freed from chloride by appropriate means, gives a very good glyoxylic reaction.

J. The reactions of certain Sclero-proteins.

Gelatin is found in the body in the form of its anhydride, collagen. This occurs in white fibrous tissue and in the organic substance of bones, and can be converted into gelatin by boiling with a dilute acid. Dried gelatin swells in cold water, but is quite insoluble in it. On warming, a more or less viscid solution is obtained, which solidifies to a jelly on cooling provided the concentration be greater than 1 per cent. This process is reversible on warming and cooling. It is precipitated by half-saturation with ammonium sulphate, by tannic acid, phospho-tungstic acid, Esbach's and Brücke's reagents, but not by normal lead acetate. On complete hydrolysis it yields a high percentage of its nitrogen in the form of glycine, but very little in the form of the aromatic amino-acids, tyrosine or tryptophane, and none as the

sulphur containing compounds exist. The tests and solutions used to test the [illegible] Millon's and sulphur colour tests for proteins and only [illegible] several slight xantho protein test which [illegible] to an impurity or to a small amount of phenylalanine.

[illegible]

a) [illegible]

b) Millon's test [illegible]

c) Glyoxylic acid test [illegible]

d) Biuret test [illegible]

e) Sulphur test [illegible] Ex. 5.

f) Add acetic acid [illegible]

g) Add acetic acid [illegible] no precipitate.

h) Add tannic acid [illegible]

i) Add lead acetate [illegible]

j) Half saturate with ammonium sulphate. Test with biuret the solution is precipitated [illegible] test on the filtrate to detect proteoses.

k) Add Esbach's or Heller's [illegible] to the filtrate to precipitate albumoses from peptones.

Keratin. **An insoluble body found in the hair, skin, nails, and horns. Remarkable for the high percentage of cystine it yields on acid hydrolysis.**

60. Perform the following tests by using horn shavings, or hair. Note insolubility in hot or cold water dilute acids, and dilute alkalies.

(*a*) Xanthoproteic reaction: well marked.

(*b*) Millon's reaction: well marked.

(*c*) Glyoxylic reaction: well marked.

(*d*) Biuret reaction: not obtained, owing to insolubility.

(*e*) Sulphur reaction: well marked.

CHAPTER II.

THE CARBOHYDRATES.

These compounds contain the elements carbon, hydrogen and oxygen, the general formula being $C_x(H_2O)_y$. They can be sub-divided into several groups.

A. The Monosaccharides.

B. The Disaccharides.

C. The Polysaccharides.

A. The Monosaccharides.

The **monosaccharides** are the simplest carbohydrates, and all the others can be hydrolysed to two or more molecules of monosaccharide by means of acids or certain ferments.

They consist of primary alcoholic ($-CH_2OH$) or secondary alcoholic ($=CH.OH$) groups linked to an aldehyde ($-CHO$) or ketone ($=C=O$) group. Those with an aldehyde group are called aldoses; those with a ketone group, ketoses. They contain from two to nine carbon atoms and are called bioses, trioses, tetroses, pentoses, hexoses, etc., depending on the number of carbon atoms in the molecule.

The lower members of the series are not important physiologically. The *pentoses* $C_5H_{10}O_5$ are found in the urine in certain pathological conditions. They form a constituent part of the molecule of nucleic acid. (See page 18.) The most important pentoses are the aldoses arabinose and xylose, obtained from gum arabic and pine-wood or straw respectively and ribose, obtained by the hydrolysis of the nucleoproteins.

[illegible] CHO [illegible]

CHO	CHO	CHO	CHOH
HCOH	HOCH	HCOH	CO
HOCH	HOCH	HOCH	HOCH
HCOH	HCOH	HOCH	HCOH
HCOH	HCOH	HCOH	HCOH
CHOH	CHOH	CHOH	CHOH

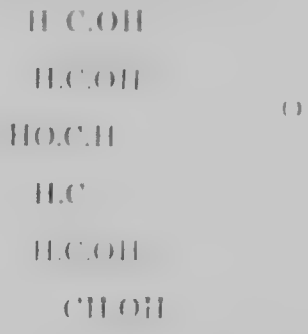
[illegible]

It will be noticed that the first three are aldoses, whilst fructose is a ketose.

The first three are stereo-isomers, differing only in the arrangement of the H and OH groups in space round the four central carbon atoms, all of which are asymmetric. (See page 79.) It therefore follows that these compounds are optically active, that is, their solutions can rotate the plane of polarised light.

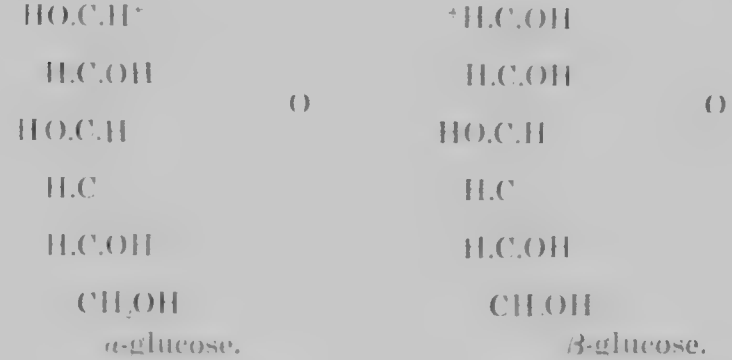
In the above formulæ they are represented as being aldehydes, but certain facts seem to indicate that they can exist in another form. Thus if glucose be dissolved in water it is found that the solution at first has a much higher rotatory power than when it has been kept for some hours or has been boiled with a trace of alkali. This phenomenon is known as mutarotation. Also it is very much less active chemically than the above formulæ warrants.

These properties are explained by assuming that when first dissolved in water, glucose exists as a γ-lactone, having the formula



In this state the *C atom is asymmetric, so that two forms of glucose are possible, called α- and β-glucose.

Under certain conditions two forms of glucose can be isolated, one with a rotary power of 110°, the other with a rotation of 19°. When kept in solution both finally attain a rotation of 52·5°.



α-glucose. β-glucose.

In solution both forms slowly pass into the aldehyde form (tautomerism). If the H atom be replaced by some other group (generally aromatic), the compound formed is called an α- or β-glucoside, which can be converted into glucose and another compound by hydrolysis with acids or certain ferments.

The natural glucosides (phloridzin, salicin, etc.) are β-glucosides.

Physical properties of the monosaccharides. They are white crystalline solids, very soluble in water and alcohol, insoluble in ether, acetone and most of the organic solvents.

They are optically active, the natural sugars having the following rotatory powers:

Glucose	+ 52·5°.	Galactose	+ 82°.
		Fructose	- 93·8°.

Chemical properties. Being aldehydes or ketones, they are susceptible of being oxidised to various acids, thus reducing certain oxidising reagents. This reaction only takes place in hot alkaline solutions, and is of great value as a test for these sugars, and especially as a basis of various methods of estimation.

They react with phenyl hydrazine in excess to give insoluble crystalline bodies called osazones. These are of the greatest value in determining the presence of and in characterising the monosaccharides, though not in distinguishing them from one another.

When heated with an alkali the monosaccharides become yellow and then brown, and finally decompose into a mixture of acids and resinous substances.

They are reduced by sodium amalgam to hexahydric alcohols. Sorbite is formed from glucose, mannite from mannose and dulcite from galactose. Fructose yields a mixture of sorbite and mannite.

On oxidation glucose gives rise to three acids:

CO_2H	CHO	CO_2H
$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$
CH_2OH	CO_2H	CO_2H
Gluconic acid.	Glycuronic acid.	Saccharic acid.

Glycuronic acid is interesting physiologically, as it is frequently found in the urine in combination with various drugs, such as chloral, camphor, phenol, etc., in the form of a glucoside. These compounds protect the organism from the injurious effects of the drugs.

Glucose (dextrose or grape-sugar). Use a 2 per cent solution for the following reactions.

Moore's test.

Treat two or three c.c. of 5 per cent. caustic soda with

$Ca(OH)_2$, is formed. Add to the mixture an equal bulk of the sugar solution. The precipitate

Cu_2O. (**Trommer's test.**

The reaction is a type of several that have been introduced for the

The property that glucose and other sugars have

7. Boil about 3 c.c. of Fehling's solution (see Note 1) in a test tube. No change occurs. Add about 3 c.c. of the glucose solution and boil again. A red precipitate of cuprous oxide is formed. (**Fehling's test.**)

NOTES—1. Fehling's fluid is prepared as follows:

(*a*) Dissolve 103.92 grams of pure copper sulphate in warm water and dilute to one litre.

[illegible] 100 grams of potassium sodium tartrate, Rochelle salt or [illegible] and 100 c.c. of caustic soda solution and dilute the whole to [illegible] with water.

[illegible] 173 grams of crystallised copper sulphate and dilute to [illegible] c.c.

For use take equal quantities of *a*, *b*, and [illegible] Though the [illegible] constituents keep indefinitely, the final mixture should be prepared [illegible] as required, and must always be boiled before using.

[illegible] a test tube and a spirit lamp at the outset of the experiment.

[illegible] Rochelle salt [illegible] to the solution of copper sulphate [illegible] the latter is precipitated [illegible]

[illegible] to the [illegible] solution.

Add the [illegible] being to boil [illegible] the mixture.

Now warm the [illegible] test tube [illegible] If the [illegible] is present [illegible] will be added [illegible] to the [illegible] a yellow, orange, or red precipitate of cuprous oxide [illegible]

[illegible] solution, owing to the excess of alkali present, to destroy the glucose before the latter can reduce the copper. The normal [illegible] should be tested with Fehling's solution and then boiled.

Test. To 5 c.c. of Benedict's solution in a test tube, add about eight drops of the sugar solution. Boil vigorously for one or two minutes and allow the tube to cool spontaneously. The entire body of solution will be filled with a precipitate, red, yellow, or green in colour depending on the concentration of the sugar. **(Benedict's test.)**

Note. [illegible] Benedict's [illegible] is a [illegible] Fehling's solution [illegible] and [illegible] carbonate [illegible] the [illegible] Fehling's solution [illegible] of [illegible] sulphate [illegible] of [illegible] crystallised copper sulphate [illegible] of water and [illegible] to [illegible] From the [illegible] solution [illegible] of Fehling's solution [illegible] The [illegible] solution does not [illegible] on standing.

Benedict's solution has certain advantages over Fehling's. For example, the test sugar is reduced by very [illegible] and can be [illegible] by chloroform, which [illegible] and so [illegible] a preservative. It does not [illegible] small amounts of [illegible] sugars

ee note 6 to previous exercise). Also it can be used for testing urines for sugar in artificial light, since it is the bulk and not the colour of the precipitate that is of importance.

69. Boil some freshly prepared Barfoed's reagent and add to it the sugar solution, drop by drop, boiling the whole time. A red precipitate of cuprous oxide is formed, either at once, or on standing for a few minutes. **(Barfoed's test.)**

NOTES.—1. The reagent is prepared by dissolving 66 gm. cupric acetate and 10 c.c. of glacial acetic acid in water and making up to 1 litre.

This test is only given by the monosaccharides, not by maltose and lactose.

3. The reagent must be freshly prepared, otherwise it is reduced by glucose and lactose.

4. Chlorides interfere with the test, causing the appearance of a greenish precipitate.

70. Boil 1 part of Nylander's solution with 5 parts of the sugar for about three minutes and allow to cool. A black precipitate of metallic bismuth settles out. **(Nylander's test.)**

NOTES.—1. Nylander's reagent is prepared by dissolving 50 gms. of Rochelle salt and 20 gms. of bismuth subnitrate in 1 litre of 8 per cent. caustic soda.

2. The reaction is of importance in detecting small quantities of glucose in urine. The uric acid and creatinine of concentrated urine reduce Fehling's solution, but have no action on Nylander's solution.

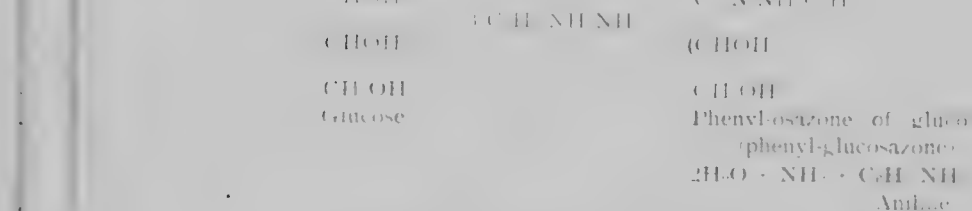
71. Treat 2 c.c. of a ·1 per cent. solution of safranine with 2 c.c. of the glucose solution and 2 c.c. of 5 per cent. sodium hydroxide. Mix and boil, avoiding any shaking. The opaque red colour gives place to a light yellow, owing to the reduction of the safranine to a "leuco-base."

72. Add to the solution of glucose some sulphindigotate of soda and some Na_2CO_3 and boil. The blue colour turns green, purplish, red, and finally yellow. Shake with air: the blue colour reappears. **(Mulder's test.)**

NOTE.—[illegible] illustrate the reducing properties of glucose [illegible] oxidation. The activity of the reduced leuco-bases for oxygen is [illegible] shaking with air.

7. Take 10 c.c. of a 1·5 per cent. solution of glucose in a test tube. Add as much solid phenyl-hydrazine hydrochloride as will lie on a sixpenny piece, and at least twice this amount of solid sodium acetate. Dissolve by warming, mix thoroughly, and filter into a clean test tube. Place this in a beaker of boiling water for at least half-an-hour, keeping the water boiling the whole time. Set the tube aside to cool (do not cool under the tap). A fine yellow crystalline precipitate of **phenyl-glucosazone** appears. Collect some of this by means of a pipette, transfer to a slide, cover with a glass and examine under both powers of the microscope. Note the characteristic arrangement of the fine yellow needles in fan-shaped aggregates, sheaves or crosses. Make a drawing of the crystals in the space provided at the end of the book.

NOTES.—Glucose is an aldehyde, and, like all aldehydes and ketones, [illegible] with phenyl-hydrazine. But this phenyl-hydrazone of glucose is very soluble, and cannot be readily separated. However, in the presence of an excess of phenyl-hydrazine at 100° C. an insoluble osazone is formed.



2. Phenyl-hydrazine is a yellow basic liquid, insoluble in water, but soluble in dilute acids to form salts. If the base itself is used, two or three drops should be dissolved in a few drops of strong acetic acid, and added to the sugar solution.

3. Phenyl-hydrazine hydrochloride, $C_6H_5.NH.NH_2.HCl$ does not give an osazone when boiled with glucose, unless an excess of sodium acetate be added. This acts on the hydrochloride to form phenyl-hydrazine acetate and sodium chloride.

B. The Disaccharides.

These carbohydrates have the empirical formula, $C_{12}H_{22}O_{11}$. They are hydrolysed by boiling with dilute

acids or by the action of certain specific enzymes into two molecules of monosaccharide.

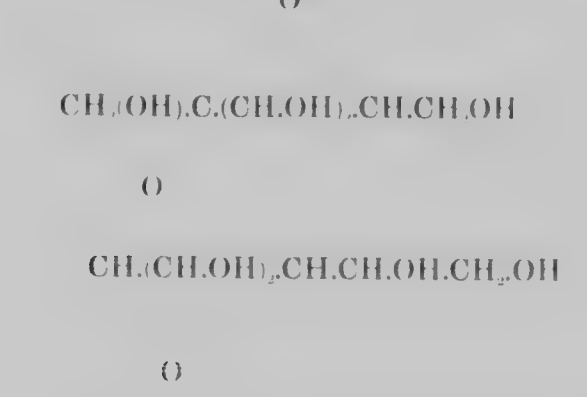
$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6.$$

The three disaccharides of physiological interest are cane-sugar, maltose and lactose (milk-sugar).

Cane-sugar (sucrose) is widely distributed in the vegetable kingdom, where it functions as a reserve material. It crystallises well, is very soluble in water, and has a much sweeter taste than glucose.

It does not reduce Fehling's solution, does not form an osazone, and does not behave as an aldelyde or ketone. It is hydroiysed very readily by boiling acids to a mixture of glucose and fructose. Cane-sugar is dextrorotatory, but since fructose is more laevorotatory than glucose is dextrorotatory, a mixture of the two in equal parts is laevorotatory. So the sign of rotation being inverted by hydrolysis, the process is known as inversion, and the product as "invert sugar." This hydrolysis is also effected by the enzyme invertase (sucrase), which is found in the small intestine and in certain yeasts.

The constitution of cane-sugar is not yet definitely established, but in all probability it is formed by the condensation of glucose and fructose in such a way as to destroy both the aldehyde and the ketone groups.



74. Repeat experiments 65, 67 and 68, with a freshly prepared 1 per cent. solution of pure white crystalline cane-sugar ("coffee sugar"). Note that it is unaffected by alkali and exerts no reducing reaction on Fehling's solution.

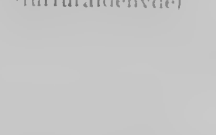
75. Treat 3 c.c. of the solution with one drop of strong sulphuric acid and boil for a minute. Add a drop of litmus solution and neutralise with caustic soda. Apply Trommer's or Fehling's test to portions of this fluid. A well marked reduction is obtained in both cases.

NOTE. This reaction depends on the fact that although cane-sugar is a non reducing sugar, it is converted to equal parts of glucose and laevulose by boiling with dilute mineral acids

$$\underset{\text{Cane sugar.}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Laevulose}}{C_6H_{12}O_6}$$

76. Treat four drops of the solution of cane-sugar with four drops of 2 per cent. solution of alpha-naphthol in alcohol and 5 c.c. of fuming hydrochloric acid. Heat to boiling point. The fluid immediately begins to assume a rich purple tint.

NOTES. - 1. This reaction depends on the fact that the laevulose, which is formed by the action of the acid on the cane-sugar, yields furfurol (furfuraldehyde)



which in its turn reacts with the alpha-naphthol to give a purple colour.

2. Glucose, lactose, and maltose only give this reaction very feebly. The polysaccharides and especially cellulose give a fair reaction. It is also given by certain proteins, when it is known as Molisch's reaction.

3. In using the reaction as a test for cane-sugar, great care must be taken to remove proteins and dextrins from solution by the method described in Ex. 55. The residue left after evaporation of the alcohol will contain all the sugars present in the original fluid.

4. Thymol can be used instead of alpha-naphthol.

77. Mix a solution of cane-sugar with one of glucose. Boil the mixture with Fehling's solution, adding the Fehling's solution to the boiling fluid until a blue colour by transmitted light indicates a slight excess of Fehling's solution. By this procedure the glucose is destroyed, but the cane-sugar is unaffected. Filter off the

precipitate of cuprous oxide. Make the filtrate acid with sulphuric and boil. Neutralise the solution, add a little more Fehling's and boil again. A well-marked reduction is obtained due to the production of glucose and laevulose by "inversion" of the cane sugar by the acid.

NOTE.—In using this as a test for cane-sugar in the presence of glucose, the presence of the polysaccharides must be excluded by alcoholic extraction if necessary: and the solution must give a well-marked alpha-naphthol test, as lactose and maltose, after boiling with Fehling's solution, give a reducing substance by acid hydrolysis

78. **Seliwanoff's test** for laevulose. Obtain a neutral solution containing laevulose as in Ex. 75. To 5 c.c. of Seliwanoff's reagent add a few drops of the sugar and heat the solution to boiling. A red colouration and a red precipitate are formed. The precipitate dissolves in alcohol, to which it imparts a striking red colour.

NOTES.—The reagent is prepared by dissolving 0·05 grm. of resorcin in 100 c.c. of 1 in 2 hydrochloric acid

The test is also given by the monosaccharides after long boiling, but a precipitate is not usually formed

Maltose is the disaccharide formed as the final product of the hydrolysis of starch by the enzyme ptyalin. It is hydrolysed by boiling acids, and by the enzyme maltose of the small intestine, to two molecules of glucose. It exhibits well-marked reducing properties towards Fehling's and Nylander's solutions, but not towards Barfoed's. It forms an osazone with phenyl-hydrazine acetate, which is more soluble than glucosazone and which melts at 206° C. Constitutionally it is glucose α-glucoside.

79. Repeat experiments 65 and 67 with a ·2 per cent. solution of maltose. It behaves like glucose.

80. Boil with Barfoed's reagent. No reduction. (Distinction from glucose.)

81. Examine microscopically and draw the crystals of phenyl-maltosazone that have been prepared by the demonstrator. Note that they are much broader than the crystals of glucosazone. Make a drawing of the crystals in the space provided at the end of the book.

Lactose is the sugar found in milk, and often in the urine of women during lactation. It has reactions very similar to those of maltose. It is hydrolysed by boiling acids, and by the ferment lactase into a molecule of glucose and one of galactose.

Constitutionally it is glucose-β-galactoside.

It is not fermented by ordinary yeast.

The osazone melts at 200° C.

82. Repeat Exs. 65 and 67 with a ·2 per cent. solution of lactose. It behaves like glucose.

83. Boil with Barfoed's reagent. No reduction. (Distinction from glucose.)

84. Examine microscopically and draw the crystals of phenyl-lactosazone that have been prepared by the demonstrator. Notice that they differ considerably from glucosazone, separating, usually, as ovoid or spherical clusters of fine needles. Make a drawing of the crystals in the space provided at the end of the book.

C. The Polysaccharides.

These compounds are formed by the condensation of more than two molecules of monosaccharides. Their general formula is $(C_6H_{10}O_5)n$.

Starch is widely found in the vegetable kingdom as a reserve carbohydrate. It occurs in the form of grains, the form of which is characteristic for a particular plant. These grains may consist of two materials, starch "granulose" and starch "cellulose", the latter forming a dense envelope to the grain; owing perhaps to this the grains are insoluble in cold water, and are only slowly attacked by enzymes. But on being boiled they absorb water and swell up to form a paste that is readily attacked by certain enzymes.

Starch has a very high molecular weight, and on being boiled with water forms an opalescent "solution" that is really a colloidal suspension. It does not diffuse through

membranes and does not depress the freezing point of water.

This so-called "starch paste" is completely precipitated by half-saturation with ammonium sulphate and by the addition of an equal volume of strong alcohol.

The most characteristic reaction of starch is the blue colour it gives with free iodine solution. It does not reduce Fehling's solution and is only slowly affected by boiling alkalies.

Starch paste is hydrolysed by boiling acids and by certain enzymes, which are therefore called the amylolytic enzymes. These are ptyalin of saliva, amylopsin of pancreatic juice and the diastases found in malt and certain yeasts and moulds.

The products of hydrolysis of starch by such a ferment as ptyalin are very numerous. The following scheme indicates the probable course of the hydrolysis, but it is not claimed that it is yet finally established.

Starch paste

Soluble Starch (Amylodextrin)

Erythrodextrin I	**Maltodextrin**	——> maltose
Erythrodextrin II	**Maltodextrin**	——> maltose
Erythrodextrin III	**Maltodextrin**	——> maltose
Achroodextrin	**Maltodextrin**	——> maltose
Achroodextrin	**Maltodextrin**	——> maltose
⋮		
Stable dextrin	**Maltodextrin**	——> maltose

Maltose. **Glucose.**

85. Place a small amount of dry potato-starch on a slide, add a drop of water, cover with a slip and examine under the microscope. Note the characteristic oval starch grains, the concentric markings and the hilum, usually eccentric. Make a drawing of the grains. Run a drop of iodine under the slip; note that the grains take on a blue colour.

86. Shake a small amount of potato starch with cold water. The starch does not dissolve. Filter, and add a drop of iodine solution to the filtrate. The characteristic blue reaction is not obtained.

87. Shake some dry starch with a little sodium carbonate solution. No change is effected. Repeat, with a little sodium hydoxide. The starch is immediately gelatinised. Add a few drops of iodine solution, a blue colour is not obtained. Treat with strong acetic acid. A deep blue colour appears.

NOTE. Free iodine is necessary to give the blue adsorption compound with starch. Sodium hydoxide removes free iodine, converting it into iodide and iodate. The action of the acid on the latter causes the appearance of free iodine and the blue colour. *Always neutralise an alkaline solution before testing for the polysaccharides.*

88. Take as much starch as will lie on a shilling, shake it up with 5 c.c. of water, and pour into 100 c.c. of boiling water, stirring the mixture during the addition. Boil for two minutes. The starch becomes gelatinised, and forms a thin, somewhat opalescent paste. Cool a portion under the tap and add a drop of iodine solution. A deep blue colour is formed.

89. Treat 5 c.c. of the cold starch paste with an equal bulk of saturated ammonium sulphate. Shake the test tube and allow it to stand for five minutes. The starch is precipitated. Filter through a dry paper, and add a drop of iodine solution to the filtrate. No blue colour, or only the very slightest tint is obtained, showing that the whole of the starch paste is precipitated by half saturation with $(NH_4)_2SO_4$.

90. Boil 5 c.c. of the starch paste with two drops of concentrated sulphuric acid for about 15 seconds. Note that the solution

becomes perfectly clear and translucent. Add two drops of strong ammonia to neutralise the acid, cool under the tap, add an exactly equal bulk of saturated $(NH_4)_2SO_4$, shake the tube vigorously and allow it to stand for five minutes. Filter through a dry filter-paper and add two drops of iodine solution to the filtrate. A deep blue colour is obtained.

NOTE.—Starch paste is rapidly converted into "soluble starch" on boiling with dilute mineral acids. Soluble starch differs from starch paste in that it is not completely precipitated by half-saturation with $(NH_4)_2SO_4$ in the course of five minutes. If it be allowed to stand for twenty-four hours, however, it is completely precipitated.

91. Take 10 c.c. of the starch paste in a small beaker. Add five drops of concentrated sulphuric acid, bring the mixture to the boiling point, and keep it boiling for seven minutes. Add a drop of litmus solution and neutralise with sodium hydroxide, keeping the reaction on the acid rather than on the alkaline side. Cool one portion under the tap and add a drop of iodine solution. A purple, red or brown reaction of erythro-dextrin is obtained, instead of the original blue reaction of starch. To the other portion add 3 c.c. of Fehling's solution and boil. A well marked reduction is obtained.

NOTE.—Starch is converted to erythro-dextrin and glucose by boiling with dilute mineral acids. If the boiling is prolonged the erythro-dextrin is converted into glucose. The extent of boiling required to destroy the whole of the starch, and yet to leave some erythro-dextrin varies with the concentrations of the starch paste and of the acid employed.

The Dextrins are polysaccharides formed by the partial hydrolysis of starch. They differ a great deal in complexity, and, with the exception of the erythrodextrins, are characterised and separated as individuals with considerable difficulty.

They all dissolve in water to form a clear solution (distinction from glycogen). They are insoluble in strong alcohol and in ether. They all reduce Fehling's solution with the exception of amylodextrin. This indicates that they contain an aldehyde or a ketone group in the

molecule. But owing to the large size of the molecule the reducing power of the higher dextrins is very slight.

Only the higher members yield a colour with iodine.

Amylodextrin gives a pure blue with iodine. It is slowly precipitated by half-saturation with ammonium sulphate: immediately by full saturation.

Erythrodextrin I. gives a purplish colour with iodine: is precipitated by full saturation with ammonium or magnesium sulphates.

Erythrodextrin II. gives a red colour with iodine. It is precipitated by full saturation with ammonium, but not by magnesium sulphate.

Erythrodextrin III. gives a red brown colour, and is not precipitated by any mixture of salts.

Achrodextrins give no colour with iodine, and are not precipitated by salts.

Maltodextrin is the name given to a substance that was separated from the mixed products obtained by the hydrolysis of starch paste by malt diastase. It consists of three molecules of maltose united together with the elimination of two molecules of water, and retaining a terminal aldehyde group. It reduces Fehling's solution, but does not ferment with yeast or give an osazone. It is hydrolysed by ferments very rapidly to maltose: by acids to glucose.

Stable dextrin is also formed by the action of amylolytic enzymes on starch paste. It is rather resistant to the action of the enzymes, but is slowly converted into a mixture of equal parts of maltose and glucose. It is formed by the condensation of forty molecules of glucose with the elimination of thirty-nine molecules of water. In the hydrolysis of starch by enzymes, about 80 per cent.

of the starch is converted into maltose, the remaining 20 per cent. being stable dextrin.

The Dextrins.

92. Shake a little commercial dextrin with some cold water. An opalescent solution is formed. Boil the solution. It becomes perfectly translucent. (Distinction from glycogen.)

Use a 5 per cent. solution of commercial dextrin for the following reactions:

93. To about 5 c.c. of the dextrin solution add iodine solution, drop by drop, noting the colour at every addition. The colour is at first almost a pure blue but it changes through a rich purple-red to a red brown as the iodine is added.

94. Repeat the above experiment, but boil and then cool the tube after each addition. The colour disappears on boiling, but does not reappear on cooling until several drops of iodine have been added.

95. Add a drop or two of the starch paste prepared in Ex. 88 to about 5 c.c. of the dextrin solution. To this mixture add diluted iodine solution, drop by drop. The first additions produce a pure blue colour, and it is not till a considerable amount of iodine has been added that the solution acquires a purplish tint.

NOTE.—The affinity of starch for iodine is so much greater than that of erythro-dextrin that the characteristic colour reactions of erythro-dextrin are not obtained until all the starch has been saturated with iodine. Even then it is sometimes difficult to detect, owing to the deep blue starch reaction.

96. Treat 5 c.c. of the dextrin solution with about 10 drops of the starch paste: to the mixture add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. The purple red reaction of erythro-dextrin is obtained.

97. Saturate 5 c.c. of the dextrin solution by boiling with an excess of finely powdered ammonium sulphate. Note the precipitate of erythro-dextrin produced. Cool under the tap and filter. To the filtrate add a drop of iodine. A red-brown colour is produced.

Note. This colour is due to the fact that erythro-dextrin III is not precipitated by ammonium sulphate. This is the method employed for the separation of erythro-dextrin in the presence of glycogen, which is completely precipitated by half-saturation with ammonium sulphate.

98. Boil a few c.c. of the dextrin solution with a small amount of Fehling's fluid. A well-marked reduction is usually obtained.

Note. Commercial dextrin is generally prepared by the action of dilute acids on starch (See Exercises 90 and 91), the action being stopped as soon a portion fails to give a blue colour with iodine, and the products then being precipitated by alcohol. Such preparations contain some dextrose, and often a little soluble starch. At the same time it must be noted that the achroo-dextrins have a reducing action themselves even when thoroughly separated from the sugars.

99. Take 10 c.c. of the dextrin solution in a small flask: add 90 c.c. of strong (95 per cent.) alcohol, place the thumb over the mouth of the flask and shake vigorously for some seconds. Note that a portion of the dextrin is precipitated as a gummy mass which sticks to the sides of the flask.

Pour off the alcohol, filter it and label the filtrate A. Rinse the flask out with a few c.c. of alcohol, shake off as much of this alcohol as possible, and add 10 c.c. of hot water. Shake this round the flask till the whole of the gummy precipitate dissolves. Divide the solution into three portions, B, C, and D. To B add a drop of iodine: a purple colour is produced. Boil C with a little Fehling's solution: only a slight reduction takes place. Boil D with two drops of concentrated sulphuric acid for two minutes, neutralise with NaOH, and boil with a little Fehling's solution: a well-marked reduction occurs.

100. To a portion of filtrate A, add a drop of iodine solution. No colour is produced. To another portion of about 5 c.c. add an equal bulk of strong alcohol. A white precipitate of achroo-dextrin is formed.

Glycogen is a reserve polysaccharide found in the liver and muscles. It forms a white amorphous powder, soluble in water to form an opalescent solution. It is

precipitated from solution by the addition of an equal volume of strong alcohol or by full saturation with ammonium sulphate. It does not reduce Fehling's solution, form an osazone or ferment with yeast. It gives a reddish colour with iodine. By boiling acids it is hydrolysed to glucose: by most of the diastatic enzymes to maltose, but by the diastase found in the liver to glucose. It is not affected by boiling alkalies. It is dextro-rotatory.

Estimation. Pflüger's method is undoubtedly the best. 20 to 100gm of the tissue is cut into small pieces and placed in an Erlenmeyer flask of Jena glass. 100 c.c. of 60% potash ("pure by alcohol" sp. gr. 1. 438) is added, a reflux condenser fitted, and the flask immersed for three hours in a boiling water bath. The alkali destroys the proteins without attacking the glycogen.

After cooling 200 c.c. of water and 800 c.c. of 96% alcohol are added and the mixture left to stand over night. The glycogen is thus precipitated free from protein. The supernatant fluid is carefully decanted and filtered. The precipitate is washed with ten times its volume of 66% alcohol, containing 1 c.c. per litre of saturated sodium chloride. After settling, the fluid is filtered through the original filter paper. This process is repeated once more, and then the precipitate is shaken with ten times its volume of 96% alcohol and filtered through the same paper. The precipitate is washed with ether, dissolved in boiling water and the solution made up to one litre. 200 c.c. of this are treated with 10 c.c. of concentrated HCl and heated in a flask on a boiling water bath for three hours, to convert the glycogen into glucose. After cooling, the solution is neutralised with 20% potash and filtered through a small paper into a 250 c.c. measuring flask. The washings from the flask used for inversion are filtered through the same paper to remove the last traces of glucose, and the solution brought up to 250 c.c. The percentage of glucose in the solution is determined by analysis. This multiplied by ·927 gives the amount of glycogen in the 200 c.c. of the solution used for inversion, and so the percentage in the tissue can be readily calculated.

Preparation. A rabbit, which has had a full meal of carrots some five or six hours previously, is killed by decapitation. The liver is cut out as quickly as possible, and the gall-bladder removed. The liver is rapidly chopped into small pieces, a small portion being reserved for Ex. 106, and the remainder immediately thrown into boiling water. After about two minutes boiling the larger morsels are strained off, pounded to a paste with sand in a mortar, and replaced in the boiling water. The proteins in solution are then coagulated by making the boiling fluid just acid with acetic acid. The fluid is filtered through coarse filter paper. In this way a crude solution of glycogen is obtained.

101. Boil 5 [illegible] test tube. The characteristic opalescence does not disappear. (Distinction from erythro-dextrin.)

102. To a small amount of the cooled solution add iodine, drop by drop. A red colour is formed, which disappears on shaking, until with a certain amount of iodine added it is permanent. Now heat the solution. The colour disappears, to reappear on cooling.

NOTE. [illegible]

103. Saturate 10 c.c. of the solution with finely-powdered $(NH_4)_2SO_4$. The glycogen is precipitated. Filter, and add a drop or two of iodine to the filtrate. No red colour is produced. Scrape the precipitate off the paper, boil with a small amount of water. The solution is markedly opalescent. Cool the solution, and add iodine. A port-wine red colour is obtained.

104. Boil 5 c.c. of the solution with a little Fehling's fluid. A very slight or no reduction is obtained.

NOTE. If the liver has been rapidly boiled, no sugar will be present. If delay has occurred during the initial stages of the preparation, some of the glycogen will have been converted into glucose. (See Exercise 106.)

105. To 10 c.c. of the solution add 20 c.c. of strong alcohol, shake vigorously and filter. To a portion of the filtrate add iodine solution. No colour is obtained, showing that the whole of the glycogen is precipitated. Dissolve the precipitate in a little hot water: note that it is opalescent. Add three drops of strong sulphuric acid and boil for about three minutes: the opalescence disappears. Neutralise with sodium hydroxide and apply Fehling's test. A marked reduction occurs, due to the conversion of the glycogen into glucose by the boiling acid.

106. The portion of rabbit's liver that was reserved has been kept in a warm place for about six hours and extracted with boiling water as before. (Or a decoction of the liver of a sheep obtained from a butcher may be used.) Note that the solution is almost

translucent. To a portion add iodine: only a very slight or no red colour at all is produced. To another portion apply Fehling's test: a well-marked reduction occurs.

107. Prepare a solution which contains equal quantities of 1 per cent. starch paste (freshly prepared), of a strong solution of glycogen and of a 3 per cent. solution of commercial dextrin. Note that the mixture is markedly opalescent.

To a small portion add diluted iodine, and note that a pure blue *starch* reaction is obtained.

To another portion of about 5 c.c. add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, leave for five minutes, and filter. Note that a portion of the filtrate gives a reddish colour with iodine, and that it is distinctly opalescent. Indication of the presence of *glycogen*.

Saturate the remainder of the fluid with finely-powdered $(NH_4)_2SO_4$ and filter. The filtrate gives a reddish-brown colour with iodine. Indication of the presence of *erythro-dextrin*.

D. The Quantitative Estimation of Sugar.

The basis of nearly all the modern methods for the volumetric estimation of the sugars is the determination of the amount of the sugar solution necessary to reduce a given volume of Fehling's solution. The chief difficulty of the original method lies in deciding the exact point when the copper is reduced, as indicated by the complete disappearance of the blue colour. This is obscured by the red precipitate of cuprous oxide that is deposited.

In Ling's method an indicator is used to determine this point. In Pavy's method strong ammonia is added to form a soluble cuprous compound. In Benedict's method potassium sulphocyanide is employed for the same purpose.

Of the methods given below, Bang's is undoubtedly the most accurate, and is to be preferred when a very reliable estimation of sugar is required.

As a standard method for general work I can strongly recommend Benedict's.

Standardisation of the Solutions. Owing to the fact that individual workers go to rather a different end point, it is advisable to perform estimations of a standard solution of sugar. This is prepared as follows: 9·5 grams of pure cane sugar are dissolved in water and the solution accurately made up to 1000 c.c. Of this solution 100 c.c. are boiled with 40 c.c. of $\frac{N}{2}$ HCl, the mixture being kept boiling for one minute. It is then cooled, neutralised by the addition of 30 c.c. of $\frac{N}{2}$ NaOH and made up to 200 c.c. with water. Such a solution contains 0·5 gm. of invert sugar per cent.

A titration of Fehling's or Benedict's solution is performed with this, and the result noted. Suppose that 10 c.c. Fehling's solution are found to be reduced by 0·054 gm. of invert sugar, use this factor rather than the theoretical 0·05.

108. **Benedict's Method.**

Principle of the Method.—An alkaline solution of copper sulphate, containing thiocyanate is boiled and the sugar solution run in from a burette till the blue colour just disappears. The thiocyanate forms a white insoluble compound with the cuprous hydroxide formed by the reduction of the copper, and so there is no red cuprous oxide precipitated to obscure the blue tint. A little potassium ferrocyanide is also added to prevent any possibility of the deposition of the cuprous oxide.

Preparation of the Solution.—With the aid of heat dissolve

Sodium citrate	...	...	200 grams.
Sodium carbonate (cryst).	...	...	200 grams.
(or anhydrous sod. carb. 75 grams.)			
Potassium thiocyanate (sulphocyanide)			125 grams.

in enough distilled water to make about 800 c.c. of the mixture and filter, and cool to room temperature.

Dissolve 18 grams of pure, air-dried crystalline copper sulphate in about 100 c.c. of distilled water, and pour it slowly into the other liquid with constant stirring. Add 5 c.c. of a 5% solution of potassium ferrocyanide and then distilled water to make the total volume 1000 c.c. The solution appears to keep indefinitely, without any special precaution, such as exclusion of light, etc.

Method of Analysis. Fit a 4-oz. flask into a ring of a retort stand of such a size that it is fairly firmly held. There is no need to use a wire gauze. Arrange the flask at such a height over a Bunsen burner that the reagent can be kept briskly boiling by means of a *small* flame. In the flask place 3 to 4 grams of anhydrous sodium carbonate. This can be roughly measured by taking a depth of 1 inch in a dry test tube. Then add 25 c.c. of the reagent and heat till the carbonate is in solution. Run the sugar solution in from a burette, which is best held in the hand. Run the sugar in at a fair rate, till a bulky chalk-white precipitate is formed and the blue colour lessens perceptibly in intensity. From this point the sugar is added more and more slowly, with constant vigorous boiling, until the disappearance of the last trace of blue colour, which marks the end-point. If the volume of the sugar used is less than 5 c.c., dilute it accurately with water till about 10 c.c. are judged necessary. Repeat the titration with this as before.

Fig. 1.

NOTES. There is a tendency to run in an excess of the sugar, unless special care is exercised throughout the titration and particularly at the end. The solution must be kept vigorously boiling during the entire process, and towards the end the sugar must be added in portions of a drop or two, with an interval of about 30 seconds after each addition. Should the mixture become too concentrated, boiled distilled water may be added to replace that lost by evaporation.

The titration can also be carried out in a white porcelain dish of 10 to 15 cm. in diameter.

Should the solution bump excessively, a *small* amount of powdered pumice-stone may be added.

Calculation of Results.

25 c.c. of Benedict's solution are reduced by 0·05 grm. of glucose
0·053 grm. laevulose.
0·074 grm. maltose
0·0676 grm. lactose

Example.—First titration required 2·4 c.c.
Solution diluted 1 in 4 (10 c.c. of sugar diluted with 30 c.c. water)
Second titration required 9·7 c.c.
So 9·7 c.c. diluted solution contain 0·05 grm. glucose

100 c.c. diluted solution contain $\frac{\cdot 05 \cdot 100}{9 \cdot 7}$

100 c.c. original solution contain $\frac{0 \cdot 05 \cdot 100 \cdot 4}{9 \cdot 7}$

Percentage of glucose 2·06

109. **Fehling's Method.**

Preparation of solution. See Ex. 67, p. 35.

Method. With a pipette measure 10 c.c. of freshly prepared Fehling's solution into a small flask. Add 40 c.c. of distilled water, heat the mixture till it boils and *keep it boiling the whole time.* Run in the sugar solution from a burette, 0·5 to 1 c.c. at a time, allowing the mixture to boil for about 15 secs. between each addition. A red precipitate of cuprous oxide forms and the intensity of the blue in the supernatant fluid decreases. Continue to add the sugar till this is completely removed. This is best determined by holding the flask by the rim at the neck and viewing it by transmitted light. If an excess of sugar be added a yellow or brown colour appears due to the formation of caramel by the action of the alkali on the sugar.

If less than 5 c.c. of the sugar are used, the solution must be diluted till about 10 c.c. are necessary. Thus if 2·5 c.c. are used in the first rough titration, the sugar should be diluted 1 in 4, by taking 25 c.c. and adding water till the volume of the solution is 100 c.c. The burette is washed out and filled with this diluted solution and the process repeated. But this time run in nearly the whole of the sugar solution judged necessary at such a rate that the mixture does not go off the boil. Then add 0·1 to 0·2 c.c. at a time till the reduction is complete. This titration should be repeated at least once more.

Calculation. 10 c.c. of Fehling's solution are reduced by 0·5 gm. glucose

Example. 1·5 c.c. of the original solution necessary
Sugar diluted 1 in 7 (10 c.c. sugar made up to 70 c.c.)
10·2 c.c. diluted sugar solution required for 10 c.c. Fehling's

10·2 c.c. dil. sugar = ·05 gm. glucose

100 c.c. " " = $\frac{05 \times 100}{10.2}$ "

100 c.c. original sugar: $\frac{05 \times 100 \times 7}{10.2}$

3·43 per cent

110. Ling's Method.

Preparation of the indicator. Dissolve 1·5 gm. ammonium thiocyanate and 1 gm. ferrous ammonium sulphate in 10 c.c. water at about 45 C. and cool at once. Add 2·5 c.c. of concentrated hydrochloric acid. The solution thus obtained has invariably a brownish-red colour, due to the presence of some ferric salt. Add zinc dust, in small portions at a time, till the fluid is just colourless. On standing for some time the red colour reappears, and must be removed again by a trace of zinc dust. But the delicacy of the indicator is impaired by being decolourised several times. When this indicator is treated with a cupric salt, the colourless ferrous thiocyanate is oxidised to the red ferric thiocyanate.

Method of analysis. 10 c.c. of Fehling's solution and about 30 c.c. of water are boiled in a flask and the sugar solution is run in from a burette as described above in Fehling's method. *The indicator is not used till the blue colour has nearly disappeared.*

Then place a drop of the indicator on a white slab. Transfer a drop of the mixture from the flask to the middle of the drop of the indicator as rapidly as possible by means of a glass tube. If a red colour appears immediately on touching the drop the reduction is not completed. More sugar must be added and a fresh drop of the indicator used as before till no colour or only a faint tinge of red is obtained. If less than 5 c.c. of the sugar solution are necessary to complete the reaction, the solution must be diluted till about 10 c.c. are required, as described above in Fehling's method.

Special precautions. Use a glass tube, not a rod, for transferring the drop.

Do not put your finger on the top of the tube. Dip it in the flask and transfer it immediately to the indicator. The flask may be taken off the boil for an instant while this is done.

Do not stir the drops on the slab.

Wash the tube before using it again.

Calculation of results. This is the same as in Fehling's method.

111. Bang's Method.

Principle. A known volume of copper thiocyanate in potassium carbonate is boiled for three minutes with a given volume of the glucose solution, that is not sufficient to reduce it completely. The copper in excess is determined by titration with hydroxylamine solution. Both the sugar and the hydroxylamine reduce the copper to colourless cuprous thiocyanate, so the end point is readily observed.

Preparation of Solutions.

1. 12·5 grams of copper sulphate are dissolved by heat in 75 c.c. of water and the solution cooled to 25° C. In a large porcelain basin 250 grams potassium carbonate, 200 grams potassium thiocyanate and 50 grams potassium bicarbonate are dissolved by stirring in 600 c.c. water. If the potassium bicarbonate does not dissolve it must be heated on the water bath to 40° C., but no higher. It is then cooled to 15° C. and the copper solution mixed with it in small quantities at a time with frequent shaking, to prevent any large amount of precipitate forming. The solution is then made up to 1 litre.

2. 6·55 grams of hydroxylamine sulphate or 5·56 grams hydroxylamine chloride are dissolved in water and the solution added to one of 200 grams potassium thiocyanate in 1500 c.c. water. The volume is made up to 2 litres.

Method of estimation. The amount of glucose added must be less than 0·06 gm. If, therefore, the solution contain less than 0·6 per cent., 10 c.c. of it are taken for the estimation. If it contain more than this, then such an amount must be taken as will yield a total amount less than 0·06 gm. In all cases the sugar solution must be made up to 10 c.c. Where there is no previous knowledge as to the strength of the sugar solution a preliminary titration should be made by boiling 10 c.c. of the sugar with 50 c.c. of the copper solution for three minutes. If the blue colour disappears,

repeat with 5 c.c., and so on until the amount is found that does not discharge the blue.

Mix the 10 c.c. sugar solution with 50 c.c. of the copper solution in an Erlenmeyer flask. Place on a wire gauze over a Bunsen burner and bring it to the boil. Maintain the boiling for exactly three minutes. Cool the solution quickly by holding the flask under the cold water tap. Titrate with the hydroxylamine solution from a burette, running it in rather slowly with frequent shaking so as to prevent any precipitate forming, which spoils the result. Add the hydroxylamine until the blue colour is discharged.

Calculation of result. The greater the excess of copper present, the greater is the reduction caused by a given weight of glucose. The reduction is therefore not proportional to the amount of sugar employed in the determination. A table has been prepared showing the weight of glucose corresponding to the amount of hydroxylamine solution that is necessary to decolourise the unreduced copper.

Example. 2 c.c. of the sugar solution and 8 c.c. of water were used. Volume of hydroxylamine required was 14·2 c.c. The table shews that 37·5 mg. of glucose were present.

So percentage of glucose is $·0375 \times \frac{100}{2} = 1·87$.

Table for calculation of amount of glucose from hydroxylamine used in Bang's method.

Hydroxylamine solution c.c.	Glucose mg.	Hydroxylamine solution c.c.	Glucose mg.	Hydroxylamine solution c.c.	Glucose mg.
43·85	5	24·10	24	10·20	43
42·75	6	23·20	25	9·50	44
41·65	7	23·10	26	8·80	45
40·60	8	22·60	27	8·50	46
39·50	9	21·75	28	7·65	47
38·40	10	21·00	29	7·05	48
37·40	11	20·15	30	6·30	49
36·40	12	19·35	31	5·90	50
35·40	13	18·55	32	5·35	51
34·40	14	17·75	33	4·75	52
33·40	15	16·95	34	4·20	53
32·45	16	16·15	35	3·60	54
31·50	17	15·35	36	3·05	55
30·55	18	14·60	37	2·60	56
29·60	19	13·80	38	2·15	57
28·65	20	13·05	39	1·65	58
27·75	21	12·30	40	1·20	59
26·85	22	11·60	41	0·75	60
26·00	23	10·90	42		

112. The estimation of Cane-sugar.

Boil 40 c.c. of the solution with 30 c.c. of $\frac{N}{2}$ hydrochloric acid keeping the mixture boiling for 1 minute. Cool, neutralise by adding 30 c.c. of $\frac{N}{2}$ sodium hydroxide, cool to 15° C. and make the volume up to 100 c.c. Estimate the amount of invert sugar in this solution by either of the methods given in the previous exercises.

Calculation of results.

25 c.c. Benedict's solution = ·0475 gm. cane sugar.
10 c.c. Fehling's „ = ·0475 gm. „ „

In Bang's method calculate as glucose and multiply by 0·95.

Estimation of Maltose and Lactose.

These are estimated by the same methods as glucose, different factors being employed for the calculation.

10 c.c. Fehling's solution } = 0·0676 gm. lactose.
25 c.c. Benedict's „ } = 0·074 gm. maltose.

CHAPTER III.

THE FATS AND THEIR DECOMPOSITION PRODUCTS.

The fats are glycerine esters of the higher fatty acids.

An ester is a compound formed by the condensation of an alcohol with an acid.

$$C_2H_5OH + HOOC.CH_3 \qquad C_2H_5OOC.CH_3 + H_2O$$

Ethyl alcohol. Acetic acid. Ethyl acetate.
(Ethyl ester of acetic acid).

Glycerine is a trivalent alcohol

$$\begin{array}{l} CH_2OH \\ | \\ CH.OH \text{ or } C_3H_5(OH)_3. \\ | \\ CH_2OH \end{array}$$

It can therefore condense with three molecules of a fatty acid.

The fatty acids found combined with glycerine are mostly palmitic acid ($C_{15}H_{31}COOH$), stearic acid ($C_{17}H_{35}COOH$) and oleic acid ($C_{17}H_{33}COOH$). The fats formed by the condensation of glycerine with these acids are known as palmitin, stearin and olein (or tri-palmitin, etc.)

$$\begin{array}{lll} CH_2OH & HOOC.C_{15}H_{31} & CH_2OOC.C_{15}H_{31} \\ CHOH & + HOOC.C_{15}H_{31} & - CH.OOC.C_{15}H_{31} + 3H_2O \\ CH_2OH & HOOC.C_{15}H_{31} & CH_2OOC.C_{15}H_{31} \end{array}$$

or $C_3H_5(OOC.C_{15}H_{31})_3$.

Palmitin.

Properties of the fats.

The fats are solids with a low melting point, triolëin melting at -5 C., tripalmitin at 65 C., and tristearin at 71 C. In the body they are found mixed in different proportions, and the melting point of the mixture is lower the greater the percentage of triolëin. They are insoluble in water, salt solutions and dilute acids and alkalies. They are soluble in ether, alcohol, chloroform and a variety of organic solvents.

They are hydrolysed by boiling acids and alkalies, by superheated steam and by certain enzymes, called lipases or steapsins. By this means they are split into their constituents, glycerine and fatty acid. If an alkali is used as the hydrolytic reagent, the fatty acid combines with it to form a soap. This special form of hydrolysis is therefore called saponification.

Various methods have been devised for the identification of the fats, amongst them being:

1. The melting point.

2. The saponification figure. A known weight of the fat is hydrolysed by means of a known amount of standard potash. The excess of alkali is then titrated, and the number of decigrams required for the hydrolysis of 100 grams of the fat is calculated.

3. The iodine number. Oleic acid is an unsaturated acid, and can combine with two atoms of iodine. The amount of iodine that combines with 100 grams of fat can be determined, and thus the percentage of unsaturated acids in the mixture calculated.

The emulsification of the fats.

Fats can be emulsified, *i.e.* broken up into droplets, either mechanically by agitation, or "spontaneously.

The mechanical emulsification is only permanent if the droplets are surrounded by a film of protein (as in milk), or by a film of soap or other more or less colloidal substance.

"Spontaneous" emulsification takes place when a melted oil or fat that contains a certain percentage of free fatty acid is brought into contact with an alkali. The fatty acid dissolves in the alkali to form a soluble soap, and the diffusion currents thus set up break the globule of fat into small particles, the process being maintained by the continual exposure of fatty acid to the alkali. The fat in the small intestine is thus emulsified as a preliminary to complete hydrolysis by the pancreatic lipase.

The digestion of fats.

The fats are hydrolysed to a small extent in the stomach by gastric lipase. This action is greater if the fat be given in an emulsified form, as in milk.

In the duodenum, the fat mixed with the fatty acid is spontaneously emulsified by the alkaline bile, succus entericus and pancreatic juice. The emulsified fat is then completely hydrolysed to glycerine and fatty acids by the pancreatic lipase. The fatty acids are converted into soluble soaps by the alkalies present. The soaps and glycerin are absorbed into the epithelial cells bordering the villi, where they are resynthesised into fats. These are passed into the lacteals and reach the blood stream by way of the thoracic duct.

113. (*a*) Carefully allow a drop of neutral olive oil to fall gently on to the surface of some ·25 per cent. Na_2CO_3 contained in a watch-glass. The drop of oil remains quite clear and forms a thin circular film on the surface.

(*b*) Shake 5 c.c. of neutral oil with 3 drops (only) of oleic acid in a dry test tube. With a drop of this mixture repeat (*a*) using a

fresh watch-glass full of Na_2CO_3. The rancid oil slowly spreads out in an amoeboid fashion and becomes converted into a milky emulsion.

(c) To the remainder of the mixture of oil and oleic acid add 12 more drops of oleic acid, shake well and repeat the experiment. The drop becomes white and opaque, but does not become emulsified.

NOTES.—1. It is absolutely essential that the oil be quite neutral, and this can best be tested by dropping it on to .25 per cent Na_2CO_3. If a spontaneous emulsion is formed, a fresh sample must be obtained, or melted fresh butter [illegible].

2. The spontaneous emulsion in (*b*) is caused by the trace of oleic acid dissolving in the alkali to form a soap, diffusion currents being thus set up which divide the fat into microscopic droplets.

3. In (*c*) the large excess of oleic acid leads to the opaque ring of soap being formed round the oil, and this soap, being only slightly soluble in water, prevents the formation of an emulsion.

114. Shake a few drops of olive oil with 5 c.c. of ether in a dry tube. The oil completely dissolves. Repeat the experiment with alcohol instead of ether. The oil dissolves partially, but is not so soluble in alcohol as in ether. Pour the alcoholic solution into water. The fat is precipitated as an emulsion.

115. Touch a piece of writing paper with a glass rod that has been dipped in olive oil. The paper is rendered translucent.

Preparation of pancreatic lipase. -A perfectly fresh pig's pancreas is freed from fat, weighed, finely minced and ground with sand. It is then treated with three times its weight of water and its own weight of strong alcohol. It is allowed to stand for three days at room temperature and strained through muslin. It must not be filtered. When not in use it should be kept in a refrigerator. It will remain active for a considerable time.

NOTE. Pancreative lipase is a ferment that only acts with the co-operation of a co-ferment, which is soluble in water and not destroyed by boiling. Bile salts and certain other substances can act as the co-ferment. The ferment proper is practically insoluble in water, and is destroyed by boiling. If the pancreatic extract be filtered, neither the precipitate nor the filtrate has any appreciable action on fats; but when the two are mixed the original lipolytic action is recovered. The precipitate is the ferment, the filtrate contains the co-ferment.

Preparation of an Emulsion of Fat.—Commercial olive oil (which contains some free oleic acid) is treated in a flask with 1 drop of a 1 per

cent. alcoholic solution of phenolphthalein for every 10 c.c. of oil. Decinormal sodium hydroxide is added, with frequent shaking, till the mixture is neutral. A very stable emulsion is thus formed, and thus a considerable surface of fat is exposed to the action of the ferment.

Fat-splitting action of lipase (steapsin).

116. Label three test tubes A, B and C.

To A add 2 c.c. of pancreatic extract and 1 c.c. of water.

" B " 2 c.c. " " boil, and add 1 c.c. of water.

" C " 2 c.c. " " and 1 c.c. of 1% bile salts.

To each add 5 c.c. of the emulsion of oil, shake thoroughly and place in a water bath at 40° C. for 1 hour.

Titrate each tube with $\frac{N}{10}$ NaOH from a burette and note the volume required to make the solution neutral. The amount required for B is a measure of the acidity of the 2 c.c. of pancreatic extract. This deducted from the amount required for A and C is a measure of the amount of fatty acid formed by the action of the ferment. It is greater in C than A, indicating the adjuvant action of bile salts on the lipolytic action.

117. Shake 5 c.c. of neutral olive oil in a test tube with 2 c.c. of the extract of the pancreas and place the tube in a water bath at 37° C. At the end of every ten minutes pipette off a little of the oil that rises to the surface, allow a drop of it to fall gently on to some 0·25 per cent. Na_2CO_3 contained in a watch-glass, return the rest to the tube, shake vigorously and return it to the warm bath. As the action of the ferment proceeds spontaneous emulsion will occur, showing that some of the neutral oil has been converted into a fatty acid. If the action is allowed to proceed considerably further no emulsion will be produced, for the reasons stated in the notes to Ex. 113.

NOTE.—This is one of the methods employed for demonstrating the fat splitting power of steapsin; but, naturally, it can only be used when perfectly neutral oil can be obtained.

118. Repeat the above experiment, but boil and then cool the 2 c.c. of pancreatic extract before adding the olive oil. A

spontaneous emulsion is not formed at any stage, showing that the ferment is destroyed.

NOTES. 1. This or a similar control experiment should always be performed side by side with the actual experiment when investigating the action of ferments.

Be particularly careful to cool the extract after boiling, otherwise the alkali may exert a slight saponifying action at the higher temperature.

119. Boil 10 c.c. of fresh milk, cool it under the tap, add 2 c.c. of the pancreatic extract, 3 c.c. of litmus solution and 2 c.c. of 2 per cent. sodium carbonate. Shake well and divide into two portions, A and B. Boil A to destroy the ferment. Place both tubes in the water bath at 37° C. In the course of ten minutes or so the blue colour in tube B will change to red, indicating that some of the neutral fat in the milk has been hydrolysed to a fatty acid.

NOTE. This is the most convenient method for the recognition of the action of lipase. The fat of milk being finely emulsified offers a very large surface for the action of the steapsin. The milk should be boiled first to destroy any bacilli present that might form lactic acid from the lactose.

Glycerine (Glycerol).

120. Treat a drop or two of glycerine in a test-tube with a solution of copper sulphate and then with sodium hydroxide. A blue solution is obtained, glycerine preventing the precipitation of cupric hydroxide.

121. Boil the solution thus obtained. Reduction does not occur.

122. Heat strongly a drop or two of pure glycerine with solid potassium hydrogen sulphate in a dry test tube. The pungent odour of acrolein (acrylic aldehyde) is noticed.

$$\underset{\text{Glycerol}}{CH_2OH.CHOH.CH_2OH} = \underset{\text{Acrolein}}{CH_2:CH.CHO} + 2H_2O$$

123. Treat about 5 c.c. of a 0·5 per cent. solution of borax with sufficient of a 1 per cent. alcoholic solution of phenolphthalein to produce a well-marked red colour. Add a 20 per cent. aqueous solution of glycerine, drop by drop, until the red colour is just

discharged. Boil the solution: the colour returns, provided that an excess of glycerine has not been added **(Dunstan's test for glycerine).**

NOTES.—1. Any ammonium salt will discharge the colour, but in this case it does not return on heating.

2. Any polyhydric alcohol is likely to give the same reaction. The sugars are all polyhydric alcohols, but are distinguished from glycerine by their reducing properties, etc., and by the fact that they are not volatile when distilled by steam.

3. The probable explanation of the reaction is as follows. Sodium borate is partially hydrolysed in aqueous solution to boric acid and sodium hydroxide. Boric acid being a weak acid is only feebly ionised and therefore the solution reacts alkaline. On adding glycerine, glyceroboric acid is formed. This is a strong acid and hence the reaction of the solution changes from alkaline to acid. On heating, unless a large excess of glycerine be present, the glyceroboric acid is hydrolysed to glycerine and boric acid and the solution again becomes alkaline.

The Higher fatty acids and their salts, the soaps.

124. Shake a few drops of oleic acid with 5 c.c. of water, ether, and alcohol respectively in separate tubes. The acid is insoluble in water, but soluble in alcohol or ether.

125. Place a drop of oleic acid on writing paper: a greasy stain results.

126. Shake the alcoholic solution of oleic acid with dilute bromine water. The colour of the bromine is discharged, owing to the unsaturated acid absorbing the halogen till it is saturated.

127. Repeat the experiment with an alcoholic solution of stearic acid or commercial "stearine" (a mixture of stearic and palmitic acids). The colour of the bromine persists, since these acids are members of the saturated series.

128. Heat about 10 drops of oleic acid with 10 c.c. of water and to the hot mixture add 40 per cent. NaOH drop by drop till the solution is clear. If an excess be added the excess of sodium ions causes a precipitate (see note below). A clear solution of a soap, sodium oleate, is formed. Divide this into three portions.

To A add a few drops of strong HCl or H_2SO_4 till the reaction is distinctly acid. Oleic acid separates out and rises to the surface of the tube.

To B add finely-powdered sodium chloride and shake. The soap is rendered insoluble and rises to the surface.

To C add some calcium chloride. A precipitate of an insoluble soap, calcium oleate, is produced.

NOTE. B illustrates the principle of "salting out," which is used in the manufacture of soaps. The excess of sodium ions in the solution, produced by the addition of the sodium chloride, lowers the solubility of the sodium oleate, which is therefore precipitated.

129. Boil 2 c.c. of olive oil with 5 c.c. of a 20 per cent. alcoholic solution of sodium hydroxide in a basin over a *small* flame for five minutes or until the alcohol has all evaporated away. Add about 5 c.c. of alcohol and heat again to dryness, stirring the whole time. Add about 30 c.c. of distilled water and boil till dissolved. Add solid sodium chloride and stir. The soap is salted out precipitated. Filter some off, dissolve in boiling water and repeat the experiments described in the previous exercise.

CHAPTER IV.

THE CHEMISTRY OF SOME FOODS.

A. Milk.

The composition of milk differs considerably in different animals.

The percentage composition of average samples of human and cow's milk is as follows:

	Protein.	Fat.	Carbohydrate.	Salts.
Human.....	1·5	3·1	5·0	0·2
Cow's.....	3·4	3·7	4·8	0·7

Other differences are that in cow's milk the proportion of caseinogen to lactalbumin is about 6 to 1 compared with 2 to 1 in human milk.

Caseinogen, the chief protein of milk, is a phosphoprotein. It is insoluble in water, dilute acids and salts, but dissolves in alkalies to form a salt-like body. It also dissolves in strong acids. It is salted out of solution by half-saturation with ammonium sulphate.

It does not coagulate on boiling. But when milk is boiled a skin forms on the surface. A similar skin forms whenever a protein solution mixed with an emulsion of a fat is heated. The skin contains protein mixed with fat. If it be removed, another skin immediately forms.

130. Examine a drop of fresh cow's milk under the microscope with a high power. Notice the highly refracted fat globules of various size, the smaller ones exhibiting the peculiar vibration known as Brownian movement.

131. Take the specific gravity of milk with a lactometer. It varies between 1028 and 1034.

NOTE.—When the milk is skimmed the specific gravity rises from 1033 to [illegible] owing to the removal of the fat which has a specific gravity of [illegible]. The specific gravity of milk is increased by dilution with water.

132. Place a drop of fresh milk on pieces of blue and red litmus paper and wash off with distilled water. The blue paper is turned red and the red paper blue, *i.e.* the milk is amphoteric in reaction, due to the mixture of acid and alkaline salts.

133. Take 5 c.c. of milk in a test tube and dilute with distilled water till the test tube is nearly full. Add three drops of dilute acetic acid and mix thoroughly. A flocculent precipitate of caseinogen is formed, which mechanically carries the fat down with it. Filter this off and label the filtrate A. Precipitate two more portions of 5 c.c. each, adding the filtrates to A, and reserving the precipitate.

134. Take 5 c.c. of milk, add water as before, and then an excess of strong acetic acid. A precipitate is not produced, owing to the solubility of caseinogen in an excess of acid.

135. Treat a portion of the precipitate from Ex. 133 with some 2 per cent. Na_2CO_3 solution. The caseinogen dissolves, leaving the fat in suspension. Apply the protein colour reaction to the solution: all, except the sulphur test, are given.

136. Treat 5 c.c. of milk with 5 c.c. of saturated ammonium sulphate solution. The caseinogen is precipitated, entangling the fat with it. Filter and boil the filtrate. A heat coagulum of lactalbumin is obtained. Treat the precipitate of caseinogen and fat on the paper with water. The caseinogen dissolves.

NOTE.—The caseinogen dissolves in water because it is precipitated as a salt by ammonium sulphate. On the addition of dilute acetic acid to the solution, a precipitate of caseinogen is again obtained.

137. Treat a considerable portion of the precipitate obtained in Ex. 133 as directed in Ex. 39. Phosphorus is found to be present in the caseinogen.

138. Allow another portion of the precipitate obtained in Ex. 133 to drain thoroughly, press it with dry filter paper and transfer it to a dry tube. Shake it vigorously with 5 c.c. of ether, pipette off the ether, and evaporate the ethereal solution in a basin over a boiling water bath, turning out the flame before putting on the dish containing the ether. A small amount of fat is left in the dish. Wipe the dish round with a piece of writing-paper. A transparent grease spot is formed.

139. Examine filtrate A. Add a drop of litmus, and note that it is markedly acid. Boil, and whilst boiling add 2 per cent. Na_2CO_3, drop by drop, until the reaction is only faintly acid. If the reaction should, by accident, be made alkaline, dilute acetic acid must be added till the reaction is faintly acid. A coagulum of lactalbumin is formed. Filter this off and reserve the filtrate (B).

140. Boil a small portion of filtrate B with a little Fehling's solution. A well-marked reduction is obtained, due to the presence of lactose.

141. Try Barfoed's reaction with this filtrate. A reduction is not usually obtained. (See Ex. 69.) Sometimes the lactose is slightly hydrolysed by the boiling in Ex. 139.

142. Treat the remainder of filtrate B with two or three drops of strong ammonia and boil. A slight precipitate of calcium phosphate is produced. Filter this off, dissolve it in a little strong acetic acid, and add a solution of potassium oxalate. A white precipitate of calcium oxalate is formed. Treat with 2 c.c. of nitric acid and 5 c.c. of ammonium molybdate solution. Boil for two minutes. A yellow crystalline precipitate is formed, showing the presence of phosphates in milk.

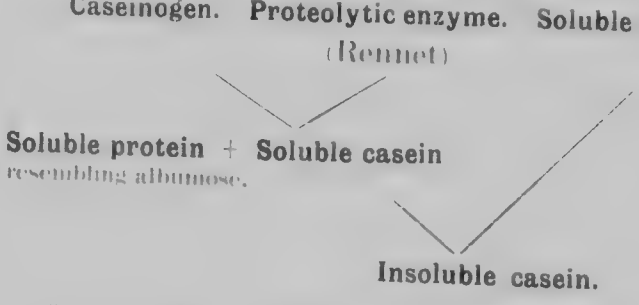
B. The Clotting of Milk.

When milk is treated with a neutral or faintly acid extract of the mucous membrane of the stomach, a clot forms after a certain time. This is due to the conversion of the caseinogen of the milk into an insoluble protein

called casein. This entangles the greater portion of the fat, the whole being known as the curd. The fluid portion that separates from the curd is called the whey, and contains the salts, lactose and lactalbumin.

The conversion of caseinogen into casein was believed at one time to be due to a special enzyme called rennet or rennin. But it is probable that the action is one common to all proteolytic enzymes, as trypsin and erepsin can cause milk to clot.

Soluble ionised calcium salts participate in the clotting action, their rôle being to convert a soluble product of ferment action into an insoluble one. The mechanism of clothing is shewn in the following scheme:



The following experiments can be performed with a commercial preparation of rennet:

143. Treat 5 c.c. of milk with about 2 c.c. of an active solution of rennet-ferment. Place the tube in the warm bath, and observe it from time to time. Note that the milk soon forms a clot so firm that the tube can safely be inverted: on standing longer the clot contracts and exudes a nearly clear fluid (whey).

144. Perform a control test by boiling and then cooling the rennet before adding it to the milk. Clotting does not take place.

145. Treat 5 c.c. of milk with 2 c.c. of 2 per cent. Na_2CO_3 and the same amount of rennet: place the tube in the warm bath. Clotting does not take place.

NOTE.—Commercial rennin is prepared from the fourth stomach of a suckling calf, or from the mucous membrane of the stomach of a pig. The pepsin, and so also the rennetic action, is destroyed by alkalies.

146. Take 10 c.c. of milk, add one-third of its volume of 1 per cent. potassium oxalate (to remove all soluble calcium salts) and divide into three equal portions which are placed in three test-tubes, labelled A, B and C.

To A add 1 c.c. of 2 per cent. calcium chloride and 2 c.c. of rennet.

To B add 2 c.c. of rennet.

To C add 2 c.c. of boiled rennet.

Place the three tubes in the warm bath for about ten minutes. Note that A clots and that B and C do not.

Boil B (to destroy the rennet) and cool the tube.

To B and C add 1 c.c. of 2 per cent. calcium chloride.

A flocculent precipitate of insoluble casein is immediately formed in B: in C there is no precipitate.

NOTE. In A there is caseinogen, rennet and $CaCl_2$
In B " " and rennet.
In C " " and $CaCl_2$
After ten minutes, B contains soluble casein, which is precipitated by the subsequent addition of $CaCl_2$.

C. Cheese.

147. Shake some grated cheese in a dry test tube with ether, and examine the ethereal solution for fat as in Exercise 138. Fat is present in considerable quantity.

148. Pound the residue from the above in a mortar with a 2 per cent. solution of sodium carbonate and filter. Acidify a portion of the filtrate. A precipitate of casein is formed, which is soluble in excess of acid. To the remainder of the filtrate apply the usual protein colour reactions: they are all obtained.

D. Potatoes.

149. Scrape the clean surface of half a potato with a pen knife, keeping the scrapings as fine as possible. Place the

scrapings in a beaker of water, stir well, and strain through fine muslin into another beaker. Allow this to stand for a few minutes and then note the white deposit of starch. Pour off the supernatant fluid and reserve it for the next exercise. Fill the beaker containing the starch with water, stir well, and again allow the starch to settle. By repeating this process of lixiviation the starch can be obtained quite pure. Examine a little microscopically and note the characteristic form of the grains (See Ex. 85). Heat a little with water, cool, and add iodine. A deep blue colour is obtained.

150. Filter the fluid A, and test portions of the filtrate for proteins by the usual colour tests. Only small quantities of protein are found to be present, the most marked reaction being Millon's.

E. Flour.

White flour from the endosperm of wheat grains contains 70 to 75 per cent. of starch, about 8 per cent. of protein and about 1 per cent. of fat. The proteins are gliadin (soluble in 70 to 80 per cent. alcohol), and glutelin (soluble in alkali). When treated with water these two proteins form a sticky mass called gluten, the viscidity being due to the gliadin. Thus grains poor in gliadin, as rice and oats, do not form dough when mixed with water.

Flour only contains glucose if germination has taken place before milling.

Whole flour is obtained from the whole of the grain, except the outer husk and outer part of the bran. It is possible that it contains something essential to growth and general nourishment. It is not quite so digestible as white flour. The bran in it stimulates the intestine and so acts as a mild laxative.

151. Mix some wheat flour with a *little* water to form a *stiff* dough. Allow this to stand for a short while, preferably at 37° C.

Wrap a piece, the size of a chestnut, in muslin, and knead it for a few minutes in a basin of water; pour the suspension [illegible]

beaker, and note the white deposit of starch grains that settles down on standing. Examine this microscopically, noting that the grains differ considerably from those of potato-starch in being smaller, circular, and with a central hilum. Make a drawing of the grains. Boil a little with water, cool, and add a drop of iodine. The deep blue starch reaction is obtained.

152. Knead the dough thoroughly under the tap until no more starch comes through the muslin. A yellowish, sticky mass, known as gluten, is left behind. Test portions of this by the usual protein colour reactions: they are all obtained, gluten being a protein.

F. Bread.

The dough formed by adding water to flour is impervious to the digestive juices. Before it can be used it has to be aerated and the gluten rendered porous.

A pure culture of yeast is mixed with warm water, flour and salt. The dough thus formed is thoroughly kneaded, and the mass kept warm for some hours. During this time the yeast cells multiply and convert some of the starch into glucose and this into alcohol and CO_2. Also the ferment of the flour called diastase becomes active and converts some of the starch into glucose. More flour is added and the process allowed to proceed for some hours longer. The gas formed causes the mass to rise. The dough is weighed out into loaves, which after being allowed to rise once more for a certain time are heated to about 232° C. for an hour and a half. The heat kills the yeast, expands the gas bubbles, and causes the outer part of the dough to become hardened by coagulating the proteins. It also converts starch into soluble starch and dextrin, thus forming the crust. The brown appearance of this is due to the conversion of glucose into caramel.

153. Take a piece of the crumb of a stale white loaf, rub it up finely and pound with cold water in a mortar. Strain and squeeze through muslin. A white fluid is obtained containing wheat-flour grains. Filter the fluid. To a portion of the filtrate add a little Fehling's solution and boil: a well-marked reduction occurs due to the presence of glucose. To another portion add iodine: a purple colour is produced, showing the presence of erythrodextrin. If very dilute iodine be cautiously added, a blue colour is produced at first, showing that a small amount of soluble starch is present.

Boil a small amount of the residue of the bread with water in a beaker, strain through muslin and filter. Cool and test the filtrate for starch and dextrin. (Ex. 96 and 97.)

154. Repeat the above exercise, using the crust of bread instead of the crumb. Note that glucose is absent or present in traces only, and dextrin and starch are present, a considerable portion of the latter existing as soluble starch and being present in the cold water extract.

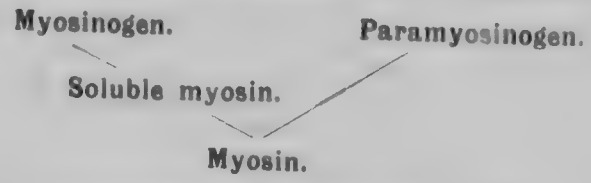
G. Meat (Muscle).

The most important constituents of living striated muscle are

Proteins.	Myosinogen and Paramyosinogen.
Pigment.	Myohaematin.
Fat.	
Nitrogenous extractives.	Creatine. Hypoxanthine. Xanthine.
Non-nitrogenous extractives.	Glycogen. Sarcolactic acid.
Inorganic.	Water. Salts, chiefly potassium and magnesium phosphates.

The **proteins** of living muscle are mainly **myosinogen** (80 per cent.) and **paramyosinogen** (20 per cent.). The former is an albumin, coagulating at 57° C. The latter is a globulin, coagulating at 47° C.

On standing or on treatment with dilute acids they are converted into **myosin** the protein of dead muscle. In this transformation, myosinogen passes through an intermediate stage of **soluble myosin** which coagulates at 40° C.



155. **Preparation of fresh muscle extract.** A rabbit is killed, a cannula fixed into the aorta and an opening made in the right auricle. The vessels are then washed free from blood with 0·9 per cent. sodium chloride. The muscles of the limbs are removed, rapidly minced and treated with ice-cold 5 per cent. magnesium sulphate, and the mixture left in the ice chest for about 24 hours. The extract is filtered and the following tests performed with it.

156. Take the reaction to litmus. It is generally neutral.

157. Dilute a small portion with four volumes of distilled water and leave the tube in the water bath at 37° C. for some time. A clot of myosin forms, leaving muscle serum.

158. Take the reaction of the muscle serum to litmus. It is distinctly acid, due to the production of sarcolactic acid.

159. Add some acetic acid to another portion of the extract. A precipitate of myosin occurs immediately.

160. Take 5 c.c. of the extract in a test-tube: place the tube in a beaker of water, supporting it by a clamp so that it does not touch the bottom of the beaker. Heat the water with a Bunsen flame and note the temperature in the tube at which distinct

coagulation occurs. It coagulates at about 63° C. Filter off the coagulated albumin and heat again. Another and larger coagulum of myosin is precipitated at 47° C.

161. **Preparation of Myosin.** Lean beef is finely minced in a machine, stirred with a large volume of water for a quarter of an hour, strained through muslin, and the washing process repeated once more. In this way certain proteins and other substances soluble in water are removed. The residue is collected on muslin, squeezed to remove the water, ground with sand, and extracted with five times its volume of 10 per cent. ammonium chloride for several hours at room temperature. The extract is filtered through muslin, linen, and finally coarse filter paper. In this way a crude, viscid solution of myosin is obtained.

162. Dilute portion of the solution with water. A heavy coagulum is formed. Wash the coagulum and on it perform the protein colour reactions. The results are recorded.

163. Pour portion of the solution into a litre of water contained in a tall cylinder; mix well, and add to the precipitate of myosin, due to the reduction in the concentration of salts.

Allow the precipitate to settle, pour off as much of the supernatant fluid as possible. Add a little of myosin dilute ammonium chloride solution to be used in the next two experiments.

[illegible]

164. To a portion add a saturated solution of common salt, drop by drop. The precipitate dissolves. Add solid NaCl to saturation: the myosin is reprecipitated.

165. To a portion add saturated $(NH_4)_2SO_4$: all the precipitate first dissolves. Now add an equal bulk of saturated $(NH_4)_2SO_4$. The myosin is reprecipitated.

166. Dissolve in a little $(NH_4)_2SO_4$, and take the temperature at which the myosin coagulates. It coagulates at about 75° C. See Exp. 160.

Creatine.—This is the most abundant nitrogenous extractive in muscle, being present to the extent of about 0·4 per cent. Chemically it is methyl-guanidine-acetic acid.

$$NH\ CH$$

$$NH_2\ C\ N—CH_2\ COOH.$$

On hydrolysis with baryta water it is converted into urea and sarcosine (methyl glycine).

$$NH\ CH_3 \qquad\qquad NH_2\ CH$$

$$NH_2C \cdot N.CH_2COOH + H_2O = \underset{\text{Urea.}}{NH_2CO} + \underset{\text{Sarcosine.}}{NH.CH_2COOH}.$$

On being boiled with mineral acids it is dehydrated to creatinine.

$$NH\ CH_2$$

$$C \cdot NCH$$

$$NH\ CO.$$

Creatinine is found in normal human urine, but creatine only under abnormal conditions.

167. **Separation of creatine from meat extract.** Dissolve 10 grams of commercial meat extract in 200 c.c. of water. Add slowly a saturated solution of lead acetate till no further precipitate is formed, carefully avoiding an excess. This is best done by filtering samples and testing them with lead acetate. Filter off the precipitate of proteins and phosphates. Warm the filtrate and decompose the soluble lead compounds by means of a stream of sulphuretted hydrogen. Warm and filter off the precipitate of lead sulphide. Evaporate the filtrate, filtering off any sulphur or sulphide that may be deposited. Continue the evaporation till a syrup is obtained. Allow this to stand in the ice chest for two or three days. Creatine separates out, mostly as oblique rhombic crystals. Examine a few under the microscope. Treat the syrup

[illegible]

[illegible] **Conversion of creatine into creatinine.** [illegible] HCl [illegible]

[illegible]

[illegible] **Jaffé's test for creatinine.** Treat 10 c.c. of the solution with [illegible] saturated picric acid solution and 5 c.c. of [illegible] per cent. caustic soda. [illegible] and dilute [illegible] formation of picramic acid from creatinine. The creatine in A gives no colour.

[illegible] **Weyl's test for creatinine.** Treat 5 c.c. with a few drops of a freshly prepared solution of sodium nitroprusside and make the solution alkaline with sodium hydroxide. A ruby-red colour appears, which soon turns yellow. Acidify with an excess of acetic acid and heat. A green tint appears, and a blue deposit of Prussian blue may result on standing.

Purine bases. These compounds are interesting because of their chemical relationship to uric acid. This relationship is shown by the formulae given on page 20.

The purine bases found in meat extracts are chiefly hypoxanthine and xanthine. They can be obtained from the alcoholic solution obtained in Ex. 166, by evaporating off the alcohol, adding ammonia and precipitating with ammoniacal silver nitrate.

Sarcolactic acid is dextro-rotatory *α*-oxy-propionic acid.

$$\begin{array}{ccc} CH & & CH \\ H-C-OH & \text{or} & HO-C-H \\ COOH & & COOH \end{array}$$

The middle carbon atom of this compound is attached to four different groups, -CH, -H, -OH and -COOH. Solutions of such asymmetric compounds have the power of rotating the plane of polarised light, either to the right or to the left.

If the carbon atom be represented as a regular tetrahedron, and the four different groups placed at the apices, then any arrangement of the groups round the tetrahedron will show a figure which is reversed by its image in a mirror. Projected on to a plane surface the above formulae are obtained. The first of these is dextro-rotatory, and the other is laevo-rotatory.

If an asymmetric compound be prepared by artificial synthesis, it consists of equal amounts of *d*- and *l*-forms, and is therefore optically inactive (racemic or *dl*-).

The lactic acid found in muscle is *d*-lactic. That formed by the fermentation of lactose and other carbohydrates is generally *dl*-lactic. Certain bacteria, however produce *l*-lactic acid.

Sarcolactic acid is present to a very small extent in fresh living muscle. The amount increases rapidly in fatigue, especially in the absence of a proper supply of oxygen. On leaving a fatigued muscle in an atmosphere of oxygen, the amount of lactic acid decreases.

There is a marked production of lactic acid at the onset of rigor mortis. But if a fresh muscle be suddenly coagulated by dropping it into boiling water, there is no such marked production of the acid.

It is probable that the lactic acid appearing in fatigue and in rigor arises through the decomposition of some complex material in the muscle, but this has not been definitely established.

Sarcolactic acid is a liquid, soluble in water, alcohol and ether. It forms a characteristic zinc salt, which is obtained by boiling a solution with excess of zinc carbonate, filtering and evaporating slowly. The crystals contain two molecules of water of crystallisation, the zinc salt of ordinary fermentation lactic acid containing three.

171. **Hopkins' reaction for lactic acid.** To 3 drops of 0·1 per cent. alcoholic solution of lactic acid in a clean, *dry* test tube add 5 c.c. of concentrated sulphuric acid and 3 drops of a saturated solution of copper sulphate. Mix and place the tube in a beaker of boiling water for about three minutes. Cool thoroughly under the tap, add two drops of a 2 per cent. alcoholic solution of thiophene, and shake. Replace the tube in the boiling water bath. As the mixture gets warm a fine cherry red colour develops.

NOTE. Lactic acid forms with sulphuric acid a substance which gives a violet colour with thiophene. The copper sulphate aids this oxidation, which is inhibited by water.

172. **Uffelmann's reaction for lactic acid.** Treat a few c.c. of Uffelmann's reagent with a few c.c. of a dilute (0·4 per cent.) solution of lactic acid. The violet colour is instantly turned to a yellow.

NOTE. Uffelmann's reagent is prepared by mixing 1 per cent. solutions of phenol (carbolic acid) with very dilute ferric chloride till the colour is changed to a greenish yellow.

Uffelmann's reaction is not reliable since other acids as tartaric, oxalic and even [illegible] give it.

173. **The Formation of Lactic Acid in Fatigue.** A pithed frog is kept on ice for about half-an-hour. Remove one hind limb and replace it on the ice. Expose the lumbar plexus of the other side and stimulate it electrically by means of a strong interrupted current for at least four minutes. Cut off the hind limb, strip the

kin off the two limbs and treat the muscles separately as follows. Rapidly remove the muscles, grind them with ice cold 95 per cent. [illegible] and sand. Transfer the mixture to a beaker, and warm for a few minutes on the water bath. Filter through a small paper and evaporate to complete dryness on a water bath. Treat the residue with about 5 c.c. of cold water and rub it up thoroughly with a glass rod. Filter and boil the filtrate with as much animal charcoal as will lie on a threepenny piece. Filter and evaporate the filtrate to complete dryness on a water bath. Allow the residue to cool and apply Hopkins' test by treating the residue with strong sulphuric acid, shaking round till solution is obtained, transferring to a dry test tube, adding three drops of saturated copper sulphate etc. A fine red colour develops in the tube containing the extract from the tetanised muscle, but none or very little in the other.

Glycogen. The percentage of glycogen in fresh muscle varies from 0·5 to 1 per cent., so that the total amount in all the muscles of the body may be greater than in the liver. The muscle glycogen decreases after muscular exercise, but not so rapidly as that in the liver.

The estimation of glycogen is described on page 49.

CHAPTER V.

THE COMPOSITION OF THE DIGESTIVE JUICES AND THE ACTION OF CERTAIN ENZYMES.

The digestive enzymes or ferments are bodies that have the power of accelerating the rate of hydrolysis of certain substances. They are often divided into groups depending on the nature of the substance on which they act (the so-called substrate or zymolyte). Thus those acting on starch are called amylolytic; on proteins, proteolytic; on fats, lipolytic, etc. The enzymes are often named in such a way as to indicate their origin and their action, the termination -ase being employed. Thus ptyalin, the amylolytic enzyme of saliva, can be termed salivary amylase, to distinguish it from pancreatic amylase (amylopsin). Gastric lipase, the lipolytic enzyme of the gastric juice, is similarly distinguished from pancreatic lipase (steapsin).

The chemical composition of the enzymes is at present uncertain, owing to the extreme difficulty of preparing them in a pure state. The proteolytic enzymes are either proteins, or compounds so readily absorbed by proteins that it is impossible to separate them. The enzymes acting on certain of the carbohydrates are possibly themselves of a carbohydrate nature.

The properties of the enzymes as a class are as follows: They are soluble in water, dilute salt solutions, dilute alcohol and glycerine. They are precipitated by saturation with ammonium sulphate and by strong

alcohol. They are colloidal and non-diffusible. They are most active at a certain temperature, called the optimum temperature, which is generally about 45° C. Their action is suspended by cooling, but is completely destroyed by raising the temperature to 100° C.

The enzymes are remarkably specific in their action, that is, they act only on a particular substance or on a group of substances having some similarity in chemical composition and configuration. A striking example of this is seen in the case of the glucosides (see page 33). The enzyme maltase (α-glucase) hydrolyses α-methyl- and α-ethyl-*d*-glucosides, but has no action on β-methyl- or β-ethyl-*d*-glucosides, or on any *l*-glucoside or on *d*- or *l*-galactosides. The enzyme emulsin (β-glucase) acts only on β-ethyl, methyl or phenyl-*d*-glucosides. Lactase acts only on the β-galactosides. It is probable that the enzyme first unites with the substrate, and to do this it must have a configuration in space corresponding with that of the substrate.

The hydrolysis is effected by the water molecules, or by the H and OH ions formed from the water. In some cases a certain concentration of H or OH ions must be present to enable the enzyme to act. Thus pepsin acts in acid solution only: trypsin requires a certain concentration of OH ions.

The action of most enzymes is retarded by the accumulation of the products of the reaction, and in certain cases the reaction is reversible.

This is well seen in the case of lipase, which induces the following reaction:

Ethyl butyrate + water $\rightleftharpoons$ ethyl alcohol + butyric acid.

The velocity of reaction is proportional to the amount of the enzyme present, provided that the amount of the

enzyme is very small compared with that of the substrate. If the amounts of enzyme and substrate are at all comparable, the laws of mass action are followed. But complications are introduced by the fact that some of the enzyme is thrown out of action by being absorbed by the products of the action.

In certain cases enzyme action is dependent on the simultaneous presence of two substances. These are sometimes called co-ferments. It has been shewn that the zymase that is responsible for the alcoholic fermentation of sugar by yeast can only act in co-operation with phosphates and some substance that is diffusible and not destroyed by boiling. Also the lipase of the pancreas requires the presence of some soluble, heat-stable substance to allow it to act. Bile salts have this property, as has been seen in a previous chapter. The action of the enzymes can be retarded by certain substances. These are of two classes: paralysers and anti-enzymes. The paralysers are generally salts of the heavy metals, which probably alter the physical state of the colloidal enzymes. The anti-enzymes are of an organic nature. They probably combine with the enzyme and thus prevent it from acting on the substrate. Examples are seen in the case of the anti-trypsin of normal serum, of the intestinal mucous membrane and of the tissues of intestinal parasitic worms.

A. Saliva.

Saliva is of value as a lubricant in the act of deglutition, and in some animals this is its sole function. In many animals, however, it contains an enzyme, ptyalin, which acts on starch, converting it finally into maltose, with perhaps a small amount of glucose. It is claimed by certain workers that for the complete hydrolysis of starch three ferments are necessary, viz., amylase that converts

starch into the dextrins: dextrinase that converts the dextrins into maltose: and maltase that converts maltose into glucose. In the case of the action of ptyalin on starch as conducted in vitro, the final product consists of about 80% of maltose, the remaining 20% being a comparatively simple dextrin called "stable dextrin," owing to its resistance to the further action of the ferment. But if this dextrin be isolated the action of ptyalin is to hydrolyse it very slowly and incompletely to equal parts of maltose and glucose.

Ptyalin acts best in a medium that is *very* faintly acid. It is rapidly destroyed by dilute HCl, but can be protected by the presence of proteins with which the acid combines, the concentration of hydrogen ions being thus decreased. It is probable that the action of ptyalin on the carbohydrates of a mixed meal continues for about 30 minutes in the mixed gastric contents.

Inorganic salts, particularly sodium chloride, favour its action, probably by causing the appearance of hydrogen ions, by some obscure absorption phenomenon of the colloidal starch. This effect of NaCl is best seen if the ferment preparation has previously been freed from electrolytes by alcohol precipitation and thorough dialysis against distilled water. Such preparations are almost inactive, but become active on the addition of traces of weak acids or neutral salts.

174. Collect about 5 c.c. of your own saliva in a small beaker. Test the reaction with neutral litmus paper: it is alkaline.

NOTE. The first portion of saliva collected is very apt to be neutral or even slightly acid, probably owing to bacterial de[illegible] [illegible] secretion is free; that collected later is invariably alkaline.

175. Transfer the saliva to a test tube and add strong acetic acid. A stringy precipitate of mucin is formed, insoluble in excess of acid. Stir the mixture vigorously with a glass rod: the mucin

[illegible]

[illegible]

In a clean test tube [illegible] prepared with distilled water [illegible]. Mix well and place the [illegible]. Place [illegible] a white porcelain plate, and from time to time transfer, [illegible] a glass rod, a drop of the digesting mixture to a [illegible]. The blue colour produced at first will later [illegible] blue violet, red-violet, red-brown, and light-brown yellow, [illegible] starch, and then the erythro-dextrin are converted into [illegible] products. When a drop of the mixture no longer gives any [illegible], boil a few c.c. of it with a few c.c. of Fehling's solution. A well marked reduction is obtained, showing that a ferment (ptyalin) in saliva has converted the starch into a reducing [illegible], however, not glucose, but maltose.

[illegible]. Perform a control test by first boiling, and then cooling [illegible] before adding it to the starch. (See Ex. 118.) No action whatever takes place when the mixture is allowed to stand on the warm bath, proving that the effect in the above exercise was due to a ferment.

[illegible]. The investigation of the activity of ptyalin under various conditions by the method of the achromic point.

In each of a series of clean test tubes place about 1 c.c. of an

[illegible] that [illegible] diluted to [illegible]

[illegible]

C[illegible] the [illegible] p[illegible] [illegible] at [illegible] C. [illegible] 40° C. [illegible]

T[illegible] starch [illegible] 5 c.c. [illegible] the [illegible] [illegible] to 40° C. [illegible] warm bath. M[illegible] the [illegible] [illegible] of the [illegible]. At intervals [illegible] a few drops [illegible] [illegible] complete [illegible] [illegible] quill tubing. [illegible] [illegible] as were seen in [illegible]. Note the time [illegible] mixture [illegible] iodine [illegible] to produce [illegible] [illegible] is the moment [illegible] the [illegible] [illegible] converted into achro[illegible] and [illegible] [illegible] achromic point. The time that [illegible] taken to reach [illegible] [illegible] period [illegible] a measure of the [illegible] of [illegible]

Repeat the exercise, and note that the [illegible] period obtained [illegible] closely with that previously found.

No[illegible] 1. It [illegible] that the starch [illegible] [illegible] [illegible] the temperature [illegible] [illegible] [illegible] strictly comparable [illegible]

2. [illegible] period is [illegible] of a[illegible] minutes [illegible] two minutes, [illegible] should be diluted with [illegible] [illegible] minutes the starch [illegible] should be [illegible] [illegible] water and boiled well to ensure [illegible] [illegible] period [illegible] new conditions [illegible] carefully noted [illegible] with those obtained in the following [illegible]

18[illegible]. Repeat the above exercise, substituting [illegible] drops of [illegible] per cent. solution of sodium chloride for the drops of water. [illegible] period is considerably reduced.

Note. The [illegible] of NaCl [illegible] [illegible] [illegible] 0.[illegible] per cent. [illegible] concentrations [illegible] [illegible] a marked [illegible] [illegible] [illegible] of NaCl [illegible]

181. Repeat the above exercise, using one drop of ·4 per cent. hydrochloric acid and four drops of water. The chromic period is considerably reduced. With two drops of ·4 per cent. HCl and three drops of water the chromic period may or may not be reduced, according to the alkalinity of the [illegible].

NOTE. One drop of ·4 per cent. HCl [illegible] centration of free HCl [illegible]

182. Repeat the above exercise, using five drops of ·4 per cent. hydrochloric acid instead of one drop. The chromic period is indefinitely prolonged.

NOTE. The concentration of HCl [illegible]

183. Repeat Exercise 179 at the temperature [illegible] 40° C. and at 55° C. The chromic period is least at 45° C.

B. Pepsin.

Pepsin is the proteolytic ferment found in the gastric juice. It acts on most proteins, finally converting them into a mixture of peptones and polypeptides. It is important to note that it does not hydrolyse them as far as free amino acids, thus differing from trypsin and erepsin. The intermediate stages in the action are given on page 24.

Pepsin acts in an acid medium only. The optimum strength of acid is one with a concentration of hydrogen ions found in a 0·2 per cent. solution of HCl. The ferment is rapidly destroyed by alkalies. It is secreted by the peptic cells of all parts of the stomach, in which it appears as a precursor, called pepsinogen. This is relatively stable to alkalies and is converted into pepsin by the action of HCl

For the following experiments use a 1 per cent. solution of commercial pepsin in water.

184. Place equal amounts of fresh washed fibrin in four test tubes labelled A, B, C and D.

To A add 5 c.c. of pepsin and 5 c.c. of ·4 per cent. HCl.

To B add 5 c.c. of pepsin and 5 c.c. of water.

To C add 5 c.c. of water and 5 c.c. of ·4 per cent. HCl.

To D add 5 c.c. of pepsin that has been boiled and then cooled, and 5 c.c. of ·4 per cent. HCl.

Place the four tubes in a water bath at 40° C. for at least thirty minutes.

Note that in

A, the fibrin swells up, becomes transparent and [illegible].

B, the fibrin is unaltered.

C, the fibrin swells up, becomes transparent, but [illegible] not [illegible].

D, the fibrin is like that in C.

NOTE.—These exercises show that neither [illegible] HCl [illegible] pepsin alone, can digest fibrin, but that pepsin in the presence of [illegible] HCl has this property. In D the ferment pepsin has been [illegible].

185. **The detection of pepsin.** Obtain some fibrin that has been stained with carmine (see note below). Treat the ferment solution with the same volume of ·4 per cent. HCl. Divide this into two equal portions and label them A and B. Boil B for a minute, and cool the tube. To each tube add a few flakes of the stained fibrin. Place them on the warm bath for ten minutes. Shake and observe the colour of the fluid. In A it will be red. In B it will be almost or quite colourless.

NOTE.—To carmine [illegible] gram of carmine in about 1 c.c. of ammonia [illegible] water. The solution is kept in a loosely-stoppered bottle till the smell of ammonia has become faint. Fresh washed fibrin is chopped finely, placed [illegible] for twenty-four hours, strained off and washed in running water till the washings are colourless. If not required immediately, it should be dried over ether and washed with water before use. It cannot be used for [illegible] pepsin, owing to the solubility of the dye in alkali.

The estimation of Pepsin by Mett's method.

Preparation of the tubes. The whites of several new-laid [illegible] are beaten to a froth, the membranes strained through muslin and the fluid allowed to stand till free from air bubbles. The albumen is then drawn up into lengths of glass tubing with an internal diameter of between 1 and 3 mm. Each length is laid flat on a piece of [illegible] arranged that it can be dropped into a saucepan of water (a strong rubber band will serve for this purpose). The water is then heated and allowed to stand till that in the inner vessel reaches 75° C. The gauze with the prepared tubes is then lifted out of the water and allowed to stand till the water has run off. The tubes can be preserved by sealing the ends with wax.

Method of estimation. Cut off lengths of 2 cms., breaking the [illegible] and place in order in a graduated glass tube.

Measure the [illegible] of the pepsin to be tested into a small Erlenmeyer flask. Dilute with [illegible] volumes of water, shake and cork, and place in the flask in a thermostat at 40° C. for 24 hours. The mixture must not be taken during the digestion. Measure the length of the tube filled with the remaining egg white (W) by means of a millimetre scale and a magnifying glass. Then W is the amount of albumen digested by P. Take the average for the three tubes. Dividing P by V gives roughly the amount of ferment present.

[illegible] $\frac{N}{10}$ HCl [illegible]

[illegible] Mett's tubes [illegible] $\frac{N}{10}$ HCl

[illegible] $\frac{N}{100}$ HCl [illegible]

Action of alkalies on Pepsin.

Treat 5 c.c. of the pepsin solution with half its volume of 2 per cent. sodium carbonate and place in the bath at 40° C. for half an hour. Neutralise with 4 per cent. HCl, and then add an equal volume of 4 per cent.

HCl [illegible] Add. A [illegible] and place the tube on [illegible]. [illegible] dissolved, showing that pepsin [illegible] dilute mineral acids.

C. The Acidity of Gastric Juice.

The acidity of the gastric contents is due to three causes, viz.:

1. The free hydrochloric acid.
2. The HCl combined with proteins.
3. Acid salts.

The sum of these three is called

4. The total acidity.

The sum of 1 and 2 is called

5. The physiologically active HCl.

The estimation of these different quantities in the gastric contents is of considerable importance in many pathological conditions. A test meal of toast and tea is given, and an hour afterwards the gastric contents are removed by means of a tube.

Total acidity. Ten c.c. of the filtered contents are titrated with N 10 NaOH, using phenolphthalein as an indicator. The result is expressed in terms of grams of HCl in 100 c.c., by multiplying the number of c.c. by 0·00365.

Free HCl. The estimation of this is practically that of the concentration of hydrogen ions in the gastric contents. HCl is very freely dissociated into H and Cl ions in such dilutions as those found in the stomach. But weak acids, as lactic and butyric, are only slightly dissociated. Also the addition of proteins to a solution of HCl decreases the concentration of H ions, owing to the formation of a compound that only dissociates to a

relatively small extent. The student is advised to read the remarks on acidity in the section on the acidity of the urine.

The estimation of the free HCl is best done by the electrical method that is mentioned in the section quoted above.

The use of indicators is not to be advised. According to the latest researches it is certain that even Toepfer's reagent (dimethyl-amido-azo-benzene) reacts with an excess of butyric and lactic acids, and also with HCl in combination with protein.

The simplest clinical method that gives results at all comparable with the electrical method is that of titrating with standard NaOH until no reaction is obtained for free HCl with Gunsberg's reagent. The method is rather tedious.

Exp. V. Gunsberg's test for free hydrochloric acid.

A. Place a single drop of Gunsberg's reagent [illegible] dish on a boiling water bath. When dry, add a single drop of 0·04 per cent. HCl to the film of reagent and again take to [illegible]. A brilliant [illegible].

B. Repeat the experiment, using a mixture of [illegible] 1 per cent. acetic acid and 1 per [illegible] HCl. Only a yellow or brown stain results.

C. To 10 c.c. of 0·04 per cent. HCl [illegible] Witte's peptone. Try Gunsberg's test with a [illegible]. Free HCl [illegible].

D. To the remainder of the fluid C add a [illegible] phthalein and titrate with N/10 NaOH till pink. Compare the amount used with that required to neutralise [illegible] 0·04 per cent. HCl. The absence of free HCl in C is obviously not due to the presence of any alkali in the peptone. The HCl [illegible] the protein to form a protein-HCl compound.

Preparation of the reagent. Dissolve 2 grammes of [illegible] and 1 gramme of vanillin in 30 c.c. of absolute alcohol. [illegible]

Estimation of free HCl by Gunsberg's reagent. If HCl is present as determined by the method given in the previous exercise, titrate [illegible] c.c. of the filtered with N/[illegible] soda, performing Gunsberg's test with a drop of the mixture after every [illegible]. The end point is reached when a drop of the mixture [illegible] give the test. If many drops have been used, the titration [illegible] be repeated, adding nearly the whole of the calculated amount [illegible] in one operation.

Calculation. Multiply the [illegible] of N/10 soda used by [illegible]. The result is the number of grams of free HCl per 100 c.c.

189. Prout-Winter method for the estimation of the physiologically active HCl and of mineral chlorides.

A. 10 c.c. of the filtered gastric contents are mixed with an [illegible] of sodium bicarbonate in a platinum crucible and evaporated to dryness over a water-bath. The crucible is then heated over a Bunsen flame and the contents incinerated. The total chlorides in the ash is determined by extracting with water and applying Volhard's method. Express the result in terms of HCl per 100 c.c.

B. Repeat the experiment without adding the bicarbonate. The free HCl and that combined with proteins is evolved, and only the mineral chlorides retained. Estimate these as before. A minus B gives the amount of physiologically active HCl.

NOTE. Usually the "active" HCl is only slightly less than the total [illegible] showing that no abnormal acids are present. But in certain diseases there is a great difference between the two results, and it is in these cases that the estimation is of value.

The amount of mineral sodium chloride is of great interest in connection with carcinoma, in which condition free HCl is absent and the mineral chlorides are much increased. This may point to a neutralisation of the acid by some alkaline secretion.

In gastric ulcer the free HCl is increased above normal, and is always considerably greater than the mineral chlorides.

D. Trypsin.

Trypsin is the proteolytic ferment secreted by the pancreas. The pancreatic juice contains a precursor called trypsinogen. This is converted into trypsin on reaching the duodenum by the action of the enterokinase secreted by the mucous membrane of the small intestine.

Trypsin differs from pepsin in two important particulars. In the first place it acts in a medium that is alkaline to litmus. The optimum concentration of hydroxyl ions is not certain. Probably that concentration in which the ferment acts best is one that has a destructive action on the ferment. Consequently the optimum concentration of alkali will be greater for a short than for a long digestion. It is important to note in this connection that trypsin is not at all stable in alkaline solutions. To preserve the ferment a minute amount of acid is added.

In the second place trypsin differs from pepsin in being able to hydrolyse the protein molecule to the final products, the various amino acids and basic substances.

Preparation of trypsin. Obtain the fresh pancreas of a dog. Free it from fat as far as possible. Weigh it. Mince it finely and add three times its weight of distilled water and its own weight of strong alcohol. Shake well in a flask and allow it to stand for three days at room temperature, shaking the flask occasionally. Strain through muslin and then through a filter folded like a pleated skirt. The filtrate, which comes through very slowly, is then acidified with 1 c.c. of strong HCl for every litre. This causes the appearance of a cloudy precipitate, which settles in a week or so and can then be filtered off. The fluid keeps for an indefinite period, if stoppered, without the addition of any antiseptic, the alcohol itself acting as an antiseptic. The fluid is rich in trypsin and in amylopsin, the amylolytic ferment of the pancreas. It does not contain any lipase.

It seems to be identical with the so-called zymogen or inactive form of the enzyme to which extract known as Benger's [illegible] pancreatics.

Detection of Trypsin.

The digestion of fibrin does not give a satisfactory method for the determination of the presence of trypsin owing to the relatively slow rate at which the action takes place.

The best method is that of Gross, who uses a solution of casein. This is precipitated by dilute acetic acid, but it is rapidly acted on by trypsin and is converted into substances that are soluble in dilute acids. We thus have a means both of detecting and of comparing the activities of tryptic solutions, by finding the time required for disappearance of a certain amount of casein.

Preparation of the Casein Solution. Dissolve 5 grams of Hammarsten casein in 12·5 c.c. of N/10 NaOH and 450 c.c. of boiling water. Filter whilst still warm, cool and make the volume up to 500 c.c.

190. Measure 10 c.c. of the casein solution into a test-tube and place it on the warm bath for a few minutes, so that it may acquire the temperature of the bath.

Measure 5 c.c. of the pancreatic extract (previously diluted with four volumes of water) into another tube and warm. Mix the two solutions, noting the time. At intervals remove about a c.c. by means of a pipette or glass tube and run it into a similar volume of 1 per cent. acetic acid. At first a heavy white precipitate of casein is produced. But after a certain length of digestion, depending on the activity of the ferment, no precipitate is produced.

NOTE.—The disappearance of the casein cannot be [illegible] HCl is not present.

The products of the action of Trypsin on Proteins.

The final products of the action of trypsin and other powerful hydrolytic reagents on proteins consist of a number of substances which differ somewhat in nature and amount with the protein. They are mostly mono-amino acids, with the amino-group replacing an H atom

attached to that carbon atom which is itself attached to the -COOH group. That is, they are *α*-amino acids.

$CH_3.CH_2.COOH$. Propionic acid.
$CH_3.CH(NH_2).COOH$. *α*-amino propionic acid.
$CH_2(NH_2).CH_2.COOH$. *β*-amino propionic acid.

Classification of the Products.

Mon-amino-acids.	Mono-carboxylic.	Fatty series.	Group A.
		Aromatic series.	Group B.
	Di-carboxylic.		Group C.
Di-amino-acids.			Group D.
Heterocyclic compounds.			Group E.
Carbohydrate compound.			Glucosamine, an amino-hexose.

Group A
1. Glycine (amino-acetic acid), $CH_2(NH_2).COOH$.
2. Alanine (*α*-amino-propionic acid), $CH_3.CH(NH_2).COOH$.
3. Leucine (*α*-amino isocaproic acid), $C_6H_{13}NO_2$.
4. Cystine (dicysteine, or di *β*-thio-*α*-amino-propionic acid).

Group B.
5. Phenylalanine. $C_6H_5.CH_2.CH(NH_2).COOH$.
6. Tyrosine (oxy-phenyl-alanine),

$$C_6H_4\begin{matrix}OH\\ \diagdown\\ \diagup\\ CH_2.CH(NH_2).COOH.\end{matrix}$$

7. Tryptophane (indol-alanine),

$$C_8H_6N.CH_2.CH(NH_2).COOH.$$

Group C.
8. Aspartic acid (amino-succinic acid).
9. Glutamic acid (*α*-amino-glutaric acid).

Group D.
10. Arginine (*α*-amino-*δ*-guanidine-valerianic acid),

$$HN:C\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ NH\end{matrix}CH_2.CH_2.CH_2.CH(NH_2).COOH.$$

11. Lysine (*α*, *ε*-diamino-caproic acid).

Group E.
12. Histidine (*β*-imidazole-alanine),

$$\begin{matrix}&&CH\\ &HN&&N\\ &&\diagup\diagdown\\ &CH&&C-CH_2.CH(NH_2).COOH.\end{matrix}$$

[illegible]

CH — CH

CH — CH.COOH

NH

[illegible]

CCH.CH.NH.COOH

CH — CH

NH.

The Isolation of the Products.

The following three methods have been employed.

1. Fractional crystallisation.
2. Fractional precipitation, that is, a reagent is used which only precipitates one or two of the substances present in the mixture, *e.g.* mercuric sulphate in acid solution only precipitates tryptophane and cystine: phosphotungstic acid only precipitates Groups D and E with the exception of proline.
3. Fractional distillation of the esters. The compounds are converted into their ethyl esters, which are dried and distilled under very low pressures. Since they have different boiling points they can be separated.

101. 150 grams of commercial casein ("protene" or "plasmon"), 50 to 100 c.c. of the tryptic solution described on page 94 and a litre of 1 per cent. Na_2CO_3 have been digested for about ten days at 40° C. in a large flask, 1 gram of sodium fluoride and about 50 c.c. of chloroform or toluol being added, and the mouth of the flask securely plugged with cotton wool, soaked in chloroform, to prevent bacterial decomposition. About 100 c.c. of the mixture are given to you. Boil the mixture, and whilst boiling add strong acetic acid, drop by drop, till the reaction is acid. Cool under the tap, and filter off the undigested casein, etc.

A. Treat 5 c.c. of the filtrate with bromine water, drop by drop: a pink colour gradually develops, which deepens and then disappears as more bromine water is added. When the colour is no longer intensified by the addition of bromine, add 2 or 3 c.c. of amyl alcohol and shake. On standing, the alcohol rises to the

CHAPTER VI.

THE COAGULATION OF BLOOD.

Factors concerned.

1. *Fibrinogen* (Fgn.) a globulin, present in blood plasma. It is soluble in dilute salt solutions, acids and alkalies, insoluble in distilled water. It coagulates at 57 C. It is precipitated by half-saturation with sodium chloride.

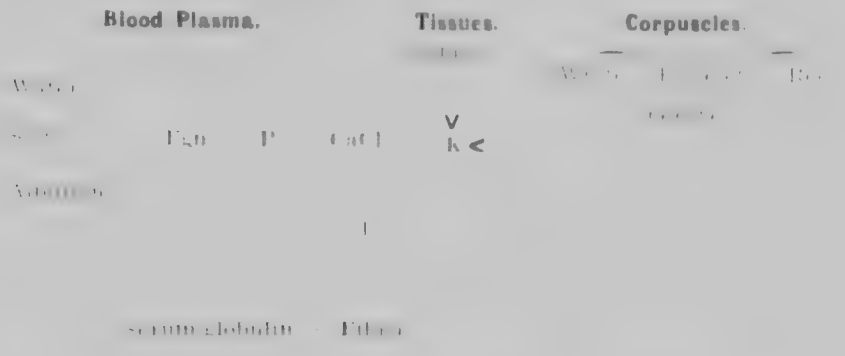
2. *Pro-thrombin* (P) a substance of unknown composition, found in plasma, attached to the fibrinogen. It is destroyed by boiling.

3. *Thrombokinase* (K) a substance found in all tissues and also liberated in the blood by the disintegration of leucocytes and blood-platelets. It converts pro-thrombin into thrombin, under certain conditions.

4. *Calcium salts*, found in plasma, and necessary for the action of thrombokinase. The calcium salts must be of such a nature that they are ionised in solution.

5. *Thrombin* (T), a ferment formed by the interaction of 2, 3 and 4. It probably splits fibrinogen into serum globulin and fibrin. The latter, being insoluble in the constituents of normal plasma, comes out of solution and with the corpuscles forms the clot.

The phenomena of blood coagulation are represented in the following scheme



Serum. Clot.

Coagulation is hindered by

1. Cooling.

2. Substances which precipitate calcium salts, or convert the calcium into the non-ionised condition, as oxalates, citrates and soap solutions.

3. Alkalies, which prevent the liberation of K by the corpuscles, delay the action of T, and tend to dissolve fibrin.

4. Strong salt solutions, which have a similar action.

5. Anti-thrombin, a substance found in small amounts in the plasma, and in relatively large amounts in extracts of the head of the leech. It combines with T to render it inactive.

6. Anti-kinase, found in the blood, after the slow injection into the blood stream of certain substances, as tissue-extracts, certain snake-venoms, etc.

7. Fluorides, which precipitate calcium salts and prevent the liberation of K.

Preparation of fibrin ferment thrombin. Blood

Preparation of "salted" plasma.

The clotting of salted plasma.

The preparation of fibrinogen.

4. Divide the solution thus obtained into two portions, C and D. To C add two drops of fibrin ferment. Place both tubes

[illegible] **The heat-coagulation of fibrinogen.** Heat [illegible] c.c. of [illegible] solution in Ex. 10. Notice the coagulation of fibrinogen [illegible] at 56° C. Continue heating to 60° C. and then filter. Dilute the filtrate as in Ex. 193; add fibrin ferment, and place on the water-bath. Coagulation [illegible].

Preparation of oxalate plasma. Blood is drawn as in the preparation of salted plasma into a bottle which has 200 c.c. of a 1 per cent. solution of potassium oxalate in place of the 100 c.c. of saturated magnesium sulphate. The plasma is separated, as before, by centrifugalizing.

[illegible] **The clotting of oxalate plasma.** Dilute 5 c.c. of the [illegible] with 5 c.c. of water, [illegible] E, F, and G. To [illegible] add [illegible] c.c. of [illegible] per cent. solution of calcium chloride. Place [illegible] the water-bath at 38° C. [illegible] F clots slowly; G [illegible].

Preparation of fluoride plasma. This is prepared [illegible] plasma with 1 per cent. solution of sodium fluoride in place of the 1 per cent. potassium oxalate.

[illegible] **The clotting of fluoride plasma.** [illegible] into two portions, H, K, and L. To H add [illegible] c.c. of [illegible] solution of calcium chloride. K [illegible] plasma ferment. Place [illegible] tubes in the water-bath at 38° C. [illegible]. H and L [illegible].

CHAPTER VII.

THE RED BLOOD CORPUSCLES AND THE BLOOD PIGMENTS.

A. The Laking of Blood.

The red corpuscles consist of an envelope and meshwork called the stroma, which encloses a solution of haemoglobin and various salts. The stroma consists of a protein, probably a histone, with which is associated a lipoid material, related to cholesterin and ecithin. The envelope behaves as a semi-permeable membrane to a great many solutions, readily allowing water to pass into or from the corpuscle, but preventing the passage of most salts and other dissolved substances. Thus if the corpuscles are placed in a solution which has a higher osmotic pressure than the fluid within the corpuscles, water passes out of the corpuscle, which therefore shrinks. Such fluids are called "hypertonic." If they be placed in fluids of a lower osmotic pressure ("hypotonic"), water passes into the corpuscle to equalise the pressures, but salts cannot pass out. The corpuscles swell and the expansion may be sufficient to lead to the disruption of the envelope, so that the enclosed haemoglobin passes into the body of the solution. This bursting of the corpuscles is known as laking or haemolysis. A solution of the same osmotic pressure as that of the fluid within the corpuscle is said to be "isotonic" or "normal." For mammalian blood 0·9 per cent. sodium chloride is normal: for frog's blood, 0·65 per. cent. Other physical means of inducing haemolysis are by repeatedly freezing and thawing the blood.

or by warming to 60° C. The envelope can also be ruptured by chemical means. Certain substances, such as the bile salts, ether, chloroform, acids, alkalies, and saponin are solvents for the lipoids.

Another method of inducing haemolysis is by the addition of certain organic substances developed in certain animals. Thus rabbit's corpuscles that have been washed with isotonic saline are laked when treated with the blood serum of a dog. This haemolytic power of dog's serum on rabbit's blood is very much increased by previously injecting the dog with rabbit's blood.

B. Haemoglobin and its Derivatives.

Haemoglobin (Hb) is a compound protein, being a member of the group of chromoproteins. It is formed by the union of a pigmented non-protein substance containing iron, and called haematin (Hm), with globin, a member of the histone group of proteins.

It is soluble in water and dilute salt solutions, insoluble in ether and alcohol.

It is decomposed by acids and alkalies into haematin and globin. It is decomposed and coagulated by heat.

It forms compounds with oxygen and carbon monoxide, called oxyhaemoglobin ($Hb\text{-}O_2$) and carboxyhaemoglobin (Hb-CO). Both are dissociated into Hb and the gas by exposure to a vacuum. Hb-CO is much more stable than $Hb\text{-}O_2$, and the avidity of Hb for CO is more than 130 times greater than the avidity of Hb for O_2. A small percentage of CO in the air breathed will thus result in the formation of relatively considerable amounts of Hb-CO in the blood. This can be converted into $Hb\text{-}O_2$ by exposure to a high tension of O_2, such as is obtained by breathing pure O_2.

The $Hb\text{-}O_2$ obtained from certain animals crystallises readily, but the crystals differ somewhat, according to the animal from which they are obtained. Also the volume of O_2 combining with 1 gram of Hb varies, the figure for the horse being 1·34 c.c. of O_2 per gram of Hb. The oxygen is probably united to the iron of the haematin molecule, the reaction $Fe + O_2 \rightleftarrows FeO_2$ being the basis of the reaction $Hb + O_2 \rightleftarrows Hb\text{-}O_2$.

The ratio $\frac{\text{volume of O, evolved in c.c.}}{\text{weight of iron in grams.}}$ is called the specific oxygen capacity.

Theoretically it is

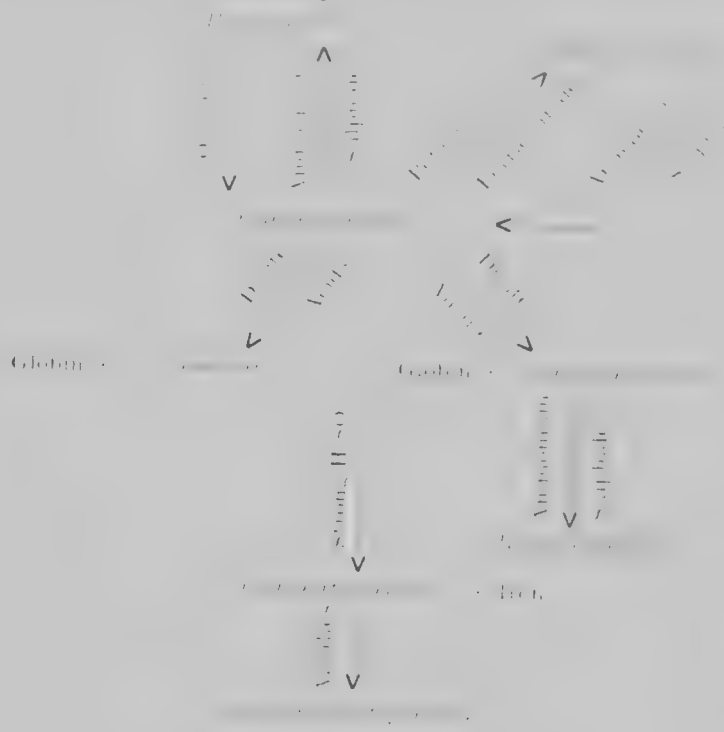
$$\frac{\text{O}}{\text{Fe}} \quad \frac{1 \text{ molecular volume O}}{1 \text{ gram molecule Fe}} \quad \frac{22{\cdot}394}{55{\cdot}85} \quad 401.$$

Recent analyses of the blood of various animals have given the value 401·8, which agrees very closely with the theoretical.

The volume of oxygen loosely held by 1 gram of Hb O$_2$ is 1·345 c.c.

So the minimum molecular weight of oxyhaemoglobin is $\frac{22{\cdot}394}{1{\cdot}345}$ 16,712.

The method of formation of certain of the derivatives of haemoglobin can be represented as follows:

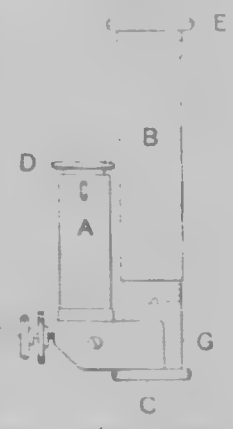


Crystallisation of oxyhaemoglobin (Rapid method).

C. The Spectroscopic Examination of the Blood Pigments.

The use of the Direct-vision Spectroscope.

The instrument described is the small pocket spectroscope, with [illegible] scale attached, manufactured by Zeiss and Co. The [illegible] (fig. 2) consists of two tubes. The shorter tube A contains a transparent photographic scale of wave-lengths, with a mirror to project its image into the field of vision. By means of the tube D this scale can be focussed and by the screw F it can be adjusted to its proper position. The tube G contains a series of alternating prisms of crown and flint glass, arranged to allow the spectrum to be observed by the eye in the line of the tube. The tube B which slides on G has a vertical slit, the width of which can be adjusted by turning the collar E.



[illegible] vision spectroscope with wave-length [illegible]

To adjust the spectroscope: see that D and B are pushed in as far as they will go. Look through C towards the light with A to your left, and turn E till the spectrum is only just visible. It is most important to use an extremely narrow slit.) Slide B out very slowly (in most instruments for 3½ divisions as marked on the barrel. Go till fine black vertical lines can be seen in the spectrum, and notice particularly a fine black line immediately to the left of the narrow strip of yellow. This line is known as the D line of Fraunhofer. The wave length of it is ·59μ, a position indicated on the scale by the division marking it (the one to the right of [illegible] than another). It may [illegible]

Oxyhaemoglobin.

dilute

Add [illegible] drop of reduced blood [illegible] ammonium [illegible] test-tube [illegible] cut off [illegible] ally [illegible] [illegible] spectroscope [illegible] ably measure [illegible] the [illegible] blood [illegible] [illegible] water between [illegible] ght [illegible] rs. Record the [illegible] solution [illegible] part [illegible] **medium** solution of [illegible] bin.

[illegible]. Add another drop [illegible] defibrinated [illegible] blood [illegible] to the blue light becomes [illegible], [illegible] only com- [illegible] ugh [illegible] d. (**Strong** solution.) If [illegible] centration [illegible] further [illegible] d, the red also is absorbed.

[illegible] It is important [illegible] of every [illegible] *dium* solution has a single band in the green.

[illegible]. **Haemoglobin (reduced haemoglobin).** [illegible] with one drop of defibrinated blood and thus obtain [illegible] haemoglobin of such a strength that two well-marked absorption bands can be observed. Add two drops of [illegible] tassium sulphide, mix and *warm to about 50° C.*, [illegible] ng the necessary shaking [illegible] or if Stokes' fluid is obtainable [illegible] two or three drops, in which case there is no necessity to warm. Note, in the latter case, that the bright scarlet colour of [illegible] oglobin gives place to the less vivid colour of reduced haemoglobin [illegible] become the solution spectroscopically. There is [illegible] e between the [illegible] in the green which overlaps the space enclosed by the two bands of oxyhaemoglobin, and is fainter than either. It is centred about λ 565.

NOTES. Stokes' fluid is prepared as follows: dissolve [illegible] of ferrous [illegible] sulphate in cold water [illegible] add a cold aqueous solution of 2 grammes of tartaric acid, [illegible] make the solution up to 100 c.c. with water. Immediately [illegible] use add strong ammonia until the precipitate first produced is redissolved. It rapidly absorbs atmospheric oxygen and must therefore be freshly prepared. The great advantage over ammonium sulphide is that it can be [illegible] the cold, [illegible] with the sulphide the solution must be warmed.

[illegible]. Place your thumb over the top of the test-tube containing the reduced haemoglobin and shake vigorously. Examine immediately with the spectroscope and note that the two bands of oxyhaemoglobin have reappeared [illegible] owing to the [illegible] the

Carboxyhaemoglobin.

Katyama's test for CO-Haemoglobin in blood.

Methaemoglobin.

16. **Acid haematin.**

17. **Acid haematin in ethereal solution.** [illegible] with the spectroscope. There is a prominent band in the red (centre λ 588); on dilution with ether three other bands can be seen: a very narrow one with centre λ 582; a broad one stretching from about λ 555 to λ 530 and another from λ 512 to λ 498.

17. **Alkaline haematin.** Treat a moderately strong solution of oxyhaemoglobin with a few drops of strong sodium hydroxide and warm. The colour changes to brown. Examine with the spectroscope: a faint band is seen in the red, stretching from the D line to about λ 630. There is a considerable absorption of the [illegible]

18. **Alkaline haematin in alcohol.** Mix defibrinated [illegible]

Haemochromogen (reduced alkaline haematin). [illegible] ne haematin from dilute oxyhaemoglobin [illegible]

Acid haematoporphyrin. [illegible]

1. **Alkaline haematoporphyrin.** To the solut[illegible] toporphyrin just prepared add five or six more drops of defibrinated blood, shaking gently after the addition of each drop. Pour the strong solution into about 50 c.c. of cold water in a beaker, [illegible] and note the precipitate that rises to the surface. Transfer this precipitate to a test tube by means of a rod; treat it with a few c.c. of alcohol and boil. Add 5 c.c. of sodium hydrate. A solution of alkaline haematoporphyrin is thus obtained, which examined spectroscopically after suitable dilutions shows a four banded spectrum. The centres of the bands are at λ 622, λ 576, λ 539 and λ 504 approximately.

Preparation of haemin crystals.

CHAPTER VIII

THE CONSTITUENTS OF BILE.

Bile is secreted continuously into the hepatic ducts by the liver. During the intervals of digestion it is stored in the gall bladder, being poured into the duodenum when the acid chyme passes through the pylorus.

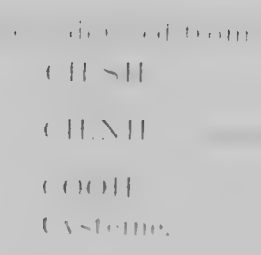
During its stay in the gall bladder there is an absorption of water and an increase in the protein content resulting in an increase in the specific gravity from about 1010 to 1040.

The percentage composition of human bile varies considerably. The following are average figures:

	From Gall Bladder.	From Fistula.
Water	86 ...	... 98
Solids	14 ...	... 2
Bile salts	9 ...	... 0·8
Protein	3 ...	... 0·3
Bile pigments		
Cholesterin	0·2 ...	... 0·06
Lecithin and fat ...	1·0 ...	... 0·01
Inorganic salts ...	0·8 ...	... 0·8

The bile salts are the sodium salts of glycocholic and taurocholic acids. They are formed by the condensation of cholalic acid ($C_{24}H_{40}O_5$) with glycine (amino-acetic acid, CH_2NH_2COOH) and taurine respectively. Glycine is one of the products obtained by the hydrolysis of proteins.

Formation of taurocholic acid and taurine.



Cholalic acid. Taurine.

The bile acids are hydrolysed into their constituents or bodies acids and also by the intestinal bacteria.

The bile salts are soluble in water and alcohol, insoluble in ether.

Their solutions have a remarkably low surface tension. See Hay's test.

They have the following functions:

1. They have a marked adjuvant action on pancreatic lipase. See Ex. 116.

2. They are solvents for the fatty acids and thus markedly increase the absorption of fats. (See p. 65.

3. They thus help to remove the fatty film surrounding the protein, and allow the proteolytic ferments to act. In this way, by assisting the absorption of proteins, they diminish bacterial decomposition. They are not direct antiseptics.

Preparation of Bile Salts. Mix 40 c.c. of ox gall with enough animal charcoal (about 10 grams) to form a paste. Evaporate to dryness over a water-bath, stirring at intervals. Grind the residue in a mortar, transfer to a flask, add about 70 c.c. of 96 per cent. or absolute alcohol and boil on the water-bath for 20 minutes. Cool and filter into a dry beaker. Add ether to the filtrate till there is a slight permanent cloudiness. Cover the beaker with a glass plate and allow it to stand in a cool place for 24 hours. A crystalline mass of bile salts separates out. The crystals are collected on a filter, washed with ether, and dried in the air.

For the following tests use a 1 per cent. solution of bile salts or diluted ox or sheep gall:

. **Pettenkofer's test for bile salts.** To 5 c.c. of the solution add a *small* particle of cane-sugar and shake: warm

Hay's test for bile salts.

Oliver's test for bile salts. [illegible]

The Bile Pigments.

Bilirubin, $C_{16}H_{18}N_2O_3$, is a reddish-brown pigment most abundant in the bile of carnivora. It is readily oxidised by the oxygen of the air into biliverdin, $C_{16}H_{18}N_2O_4$, the green pigment found mostly in the bile of herbivora. These compounds are formed in the liver cells from the products of disintegration of haemoglobin. Haematin is $C_{32}H_{32}N_4O_4Fe$, and haematoporphyrin is isomeric with bilirubin.

They are weak acids, forming sodium and calcium salts, the latter being insoluble in water. Free bilirubin is soluble in ether and chloroform: the sodium compound is insoluble, as is free or combined biliverdin.

By oxidation bilirubin is converted, through a number of ill-defined bodies, such as bilicyanin, and bilifuscin, into choletelin, the end product of Gmelin's reaction.

By further oxidation a compound, haematinic acid ($C_4H_5O_3$), is formed, identical with the product obtained by the oxidation of haematin or haematoporphyrin.

By reduction with sodium amalgam in alcoholic solution the bile pigments are converted into hydrobilirubin, which is also formed by the action of more powerful reducing reagents on haematin or haematoporphyrin.

These facts all indicate the close relationship between haematin and the bile pigments.

In the bowel the bacteria first reduce bilirubin to hydrobilirubin. This is then attacked, two nitrogen atoms being probably removed, the result being the formation of stercobilin, which is mainly excreted in the faeces. But a small amount is absorbed and excreted in the urine as urobilinogen.

(1) **Gmelin's test for bile pigments.** [illegible] nitric acid in a test tube and by means of a pipette, [illegible] place on the surface of this an equal amount [illegible] tube very gently from side to side, and note the play of [illegible] in the bile as it becomes oxidised by [illegible]. Proceeding [illegible] to bile the colours are yellow, red, violet, blue, and green.

[illegible] This test can be modified in [illegible]

[illegible] Add a drop of yellow nitric acid to a thin film of bile on a white [illegible]. The drop of acid becomes surrounded [illegible]

[illegible] [illegible] en place a drop of fuming nitric acid on the paper [illegible]

(2) **Cole's test for bile pigments.** To about 50 c.c. of [illegible] bile add an excess of barium chloride. Stir well, and [illegible] to stand for a short time. The precipitate, containing an insoluble barium compound of bilirubin, coheres together. Remove the main mass of the fluid by means of a pipette, and then filter. Open the filter paper on a tile and scrape the precipitate off the paper. Place it in a test tube, add about 4 c.c. of strong alcohol, two drops of strong sulphuric acid, two drops of a 5 per cent. solution of potassium chlorate, and boil for a minute. Allow the precipitate of barium sulphate to settle. The supernatant alcohol is coloured a greenish-blue.

The Protein of Bile.

When bile is treated with acetic acid a precipitate is formed insoluble in excess. This was formerly thought to be mucin. But it has been shown that it is nucleoprotein, the bile salts present preventing the re-solution in strong acetic acid. (See Ex. 225.) In human bile, however, mucin is present as well as nucleoprotein.

The protein is secreted by the cells lining the duct and the gall bladder, so that bile from the gall bladder contains a much greater percentage than fistula bile.

[illegible] This precipitate [illegible]

Cholesterin. $C_{26}H_{43}OH$ or $C_{27}H_{45}OH$ is a monovalent alcohol found in the bile. It is present in nearly all the fluids and tissues of the body, notably in the central nervous system. It is found in large amounts in egg yolk. In the blood plasma it is present as an ester, as it is in lanoline, the "fat" obtained from sheep's wool. We have already seen that it is a constituent of the envelope of red blood corpuscles (p. 103). It forms one of the varieties of gall stones, found after inflammation of the mucous membrane of the gall bladder.

It is soluble in ether, alcohol, chloroform, and acetone. It is only slightly soluble in cold, easily in hot alcohol. It is soluble in bile salts, insoluble in water, weak acids and alkalies. It crystallises from boiling alcohol in plates of a characteristic shape: from the other solvents in needles. It melts at 145 C., and in chloroform solution shows an optical activity [illegible].

Its chemical constitution is not yet determined, but it probably belongs to the terpene series.

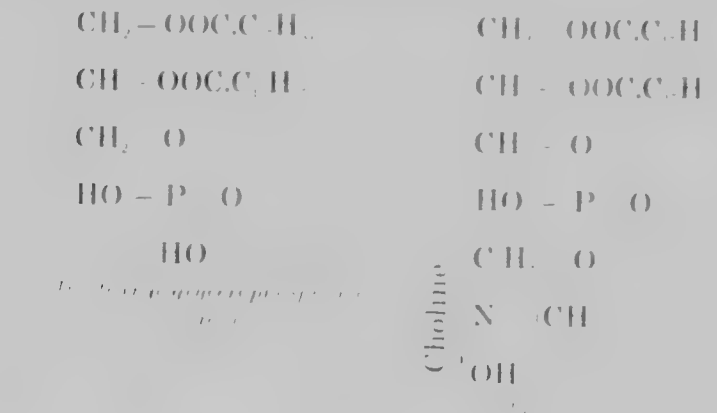
Preparation [illegible]

Salkowski's reaction for cholesterin. [illegible]

Liebermann-Burchard reaction for cholesterin. [illegible]

Lecithin is a complicated fat-like body, generally found in the body and elsewhere with cholesterin. (See p. 121.)

It can be regarded as a compound of the base choline with esters of glycerophosphoric acid.



CHAPTER IX.

URINE AND ITS CHIEF CONSTITUENTS.

A. The average composition.

The composition of the urine varies with the individual and with the diet. Below are given the figures in grams for the daily output of

A. The average man on the average mixed diet.

B. An individual on a liberal diet.

C. The same individual on a diet deficient in proteins.

B. and C. are taken from a paper by Folin.

	A.			B.			C.		
		Nitrogen	Per cent. of Total N. or %		Nitrogen.	Per cent. of Total N. or %		Nitrogen.	Per cent. of Total N. or %
Urea	30	14	87·5	31·6	14·7	87·5	4·72	2·2	61·7
Ammonia	0·6	0·5	3·1	·6	0·49	3·0	·51	0·42	11·3
Creatinine	1·55	0·57	3·6	1·55	0·58	3·6	1·61	0·60	17·2
Uric Acid	0·8	0·23	1·4	·54	0·18	1·1	·27	0·09	2·5
Undetermined		0·7	4·4		0·85	4·8		0·27	7·
Total N		16·0	100·0		16·8	100·0		3·6	100·0
Inorganic SO_3	2·92		88·2	3·27		90·0	0·46		60·5
Ethereal SO_3	·22		6·6	0·19		5·2	0·10		13·2
Neutral SO_3	·17		5·2	0·18		4·8	0·20		26·3
Total SO_3	3·1		100·0	3·64		100·0	0·76		100·0

B. The Physical Chemistry of the Urine.

I. General Properties.

Normal human urine is a clear yellowish fluid, the depth of the tint depending largely on the concentration. On standing, a cloud (nubecula) of mucoid containing epithelial cells separates out. After a heavy meal urine may be passed cloudy, due to earthy phosphates and carbonates. On standing, these settle to the bottom of the vessel as a white deposit, insoluble on warming, but soluble in acid.

Also on standing a cloud of urates may settle as a reddish deposit that clears up on warming.

Fresh urine has a characteristic odour of the aromatic type, due to the presence of some substance that has not yet been recognised. On standing, an unpleasant ammoniacal odour develops as the result of bacterial decomposition.

II. The Specific Gravity.

Usually lies between 1012 and 1024 (water 1000). With copious drinking it may fall to 1002. After excessive perspiration it may rise to 1040.

The determination of the specific gravity for clinical purposes is most conveniently made by means of a urinometer, a weighted cylinder that floats in the urine. The depth to which it sinks depends on the density of the fluid, and this can be read directly by means of a graduated scale on the stem. The instrument is calibrated for a certain temperature, usually 15° C.

The urine should be either cooled or warmed to this temperature, or a correction made by adding 1 unit for every 3 degrees above this, or subtracting 1 for every 3

degrees below the standard. Thus if the reading be 1018 at 18° C., the corrected Sp. Gr. is 1019.

To obtain the best results two separate instruments should be at hand, the one calibrated from 1000 to 1020 and the other from 1020 to 1040.

[illegible] the gravity by Long's coefficient. The last two figures of the [illegible] gives total solids in 1000 c.c.

[illegible] the gravity at 25° C. [illegible]

Total solids in 1000 c.c. 17 + 24 = 41 [illegible]

Haser's coefficient 2.33 on a similar basis, but calculated for 15° C. [illegible] probably inaccurate.

[illegible] a single [illegible] the surface. Take care that [illegible] the sides of the vessel. Place the [illegible] the surface of the fluid and read the [illegible] to which the latter reaches. Read the level of the true surface of the urine, [illegible] around the stem.

[illegible]
Urinometer

III. The Osmotic Pressure (Cryoscopy).

The freezing point of pure water is 0° C. That of solutions is lower than this, and the depression of the freezing point is proportional to the molecular concentration of the solution. In the case of electrolytes (salts, alkalies and acids) in aqueous solution it is proportional to the concentration of (molecules + ions), that is to the osmotic concentration.

Since the osmotic pressure of a solution is also proportional to the molecular or osmotic concentration of the solution, it follows that a determination of the depression of the freezing point (cryoscopy) enables us to get a measure of the osmotic pressure.

With non-electrolytes the gram-molecule in 1000 gms. of water causes a depression (Δ) of the freezing point of 1·85° C.

So that $\frac{\Delta}{1.85}$ = molecular concentration.

With electrolytes, $\frac{\Delta}{1.85}$ = osmotic concentration = concentration (molecules + ions).

The quantitative relationship between Δ and osmotic pressure is that a Δ of 0·001° C. = an osmotic pressure of 0·1 mm. mercury.

In urine the concentrations of certain substances, such as urea, are much greater than they are in the blood. The work done by the kidney in effecting this concentration can be calculated from a consideration of the osmotic concentration, *i.e.* Δ, of each substance in blood and urine. It is quite erroneous to imagine that the work done can be calculated from a knowledge of the total osmotic concentration of the blood and urine respectively.* But, at

* A full discussion of the subject will be found in Moore's article in "Recent Advances in Physiology" (p. 159).

the same time, the determination of Δ of the blood and of the urine secreted by each kidney in certain renal diseases may give us valuable information as to the relative activities of the two organs.

Δ of blood is about 0·55 C., the same as that of a 0·9 per cent. solution of sodium chloride.

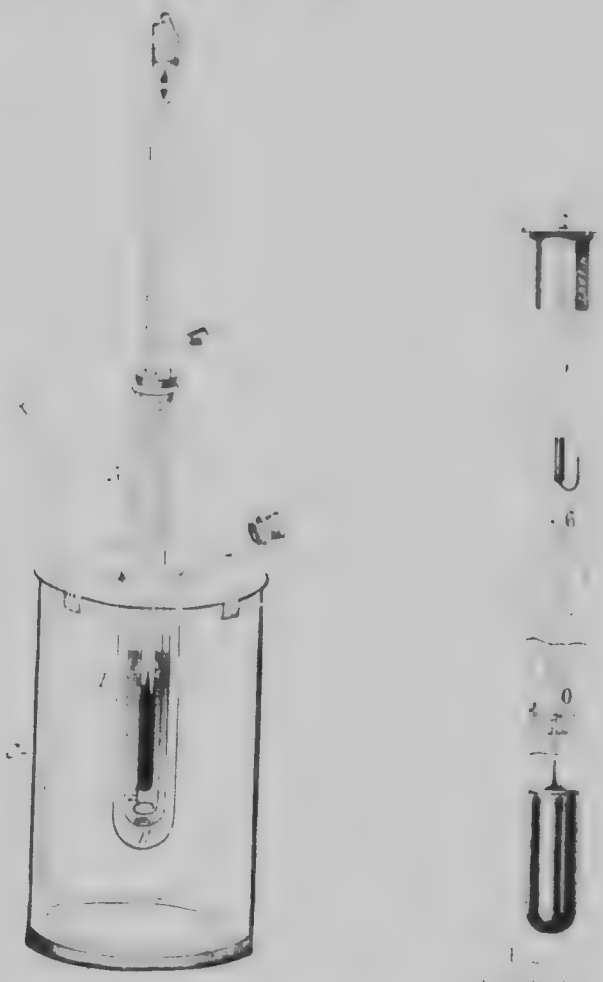
Δ of urine varies considerably with the diet, volume of fluid taken and other conditions. For the mixed 24 hours urine of an average man it is usually about 1·2 C. The following values are of interest in this connection

Δ ÷ volume of urine = molecular diurests.

$\frac{\Delta}{\text{NaCl per cent.}}$ is of considerable pathological significance. It is fairly constant in health, varying between 1·25 and 1·6. It exceeds 1·7 in heart disease or in any condition that causes a retardation of the renal circulation. The only febrile condition in which it is less than 1·7 is malaria.

The determination of the freezing point by Beckmann's method. In the outer chamber (C) place a mixture of ice and water. Add saturated salt solution until the temperature falls to about 3° C. lower than the anticipated freezing point of the urine. During the course of the experiment the freezing mixture must be stirred occasionally by means of F, and ice or salt added to maintain the temperature within about 1° C. of the desired.

In the tube A place enough distilled water to cover the bulb of the Beckmann thermometer D. This is graduated to 1/100th C., and can be read by means of a magnifying glass to 1/1000 C. The thermometer must not touch the sides or bottom of the tube A. The tube B serves as an air jacket to A. Stir the water regularly by means of the platinum stirrer E. The temperature falls, and then after a time rises sharply, and remains steady for a con-



It has been shewn that in all aqueous solutions the product of the concentration of hydrogen ions (C_H) and that of the hydroxyl ions (C_{OH}) is constant. That is $C_H \times C_{OH}$ = a constant.

In distilled water at 18° C. these concentrations are equal and are both $10^{-7.03}$. So that the constant = $10^{-7.03} \times 10^{-7.03} = 10^{-14.06}$. In solutions of acids C_H exceeds $10^{-7.03}$ and C_{OH} is less than $10^{-7.03}$, but the product of the two is always $10^{-14.06}$.

Acids differ markedly in the degree to which they are ionised in solution. Thus in N/10 hydrochloric acid 91 per cent. of it is ionised. So C_H is 0·091 N. Now 0·091 = 9·1 $\times 10^{-2} = 10^{0.96} \times 10^{-2} = 10^{-1.04}$.

It is convenient to express this as $p_H = 1{\cdot}04$. That is, p_H is the logarithm to the base 10 of the concentration of

H ions in grams per litre, the negative sign being understood.

N/10 acetic acid is only dissociated to the extent of 1·3 per cent.

So C_H is ·0013 N $\quad 1{\cdot}3 \times 10^{-2} \quad 10^{-4} \times \quad 10^{-3}$.

That is p_H $\quad 2{\cdot}89$.

An "indicator" is a substance that shows a change in colour when a certain amount of an acid or an alkali is added to it. At a certain stage of the addition there is an intermediate tint, and the solution is now said to be "neutral to that indicator." It must be clearly understood that this so-called neutrality does not necessarily correspond to an equality in the concentration of h and oh ions. Further, a solution that is neutral to one indicator may have a concentration of h ions widely different from that in a solution that is neutral to another indicator. Thus a solution neutral to phenolphthalein has a p_H about 9; one neutral to methyl-orange has p_H about 4. The value p_H for any solution can be determined electrically by means of the potential set up between the solution and hydrogen. Further, it has been shown that if two solutions show the same tint with a given indicator at about the neutral point of this indicator, then these solutions have the same p_H. Sörensen has evolved a method of determining the true acidity of solutions based on this principle. The p_H is roughly found by the addition of various indicators. Then a series of solutions is prepared with known values of p_H. A certain indicator is added to each and to the solution. Those that have exactly the same tint have equal values of p_H.

For the details of the application of the method to urine, the student should consult a paper by G. S. Walpole, Bio-chemical Journal, Vol V., p. 207.

The range of certain indicators is given below.

	p_H		
Methyl violet	0·1	-	3·2
Tropaeolin OO	1·4		2·6
Di-methyl-amino-azo-benzene (Töpfer's reagent)	2·9		4·2
Methyl Orange	3·1		4·4
Methyl Red	4·2		6·3
p-Nitrophenol	5·0		7·0
Litmus	5·0		8·0
Neutral Red	6·8	-	8·0
Rosolic Acid	6·9		8·0
α-Naphtholphthalein	7·3		8·7
Phenolphthalein	8·3		10·0
Thymolphthalein	9·3	-	10·5
Tropaeolin O	11·1	-	12·7

Normal urine has p_H about 5, that is, it is acid to litmus and phenolphthalein, but alkaline to methyl orange.

The amount of N 10 sodium hydroxide that must be added to make the mixture neutral to phenolphthalein is sometimes called its "acidity." It would be better to call this the "titration acidity." For the method of its determination see Ex. 317.

The acidity of normal urine is due partly to the presence of acid phosphates, but largely to free organic acids.

C. The Pigments of Urine.

Urochrome is the chief pigment of normal urine. It is a yellow substance which has no definite absorption band. Nothing certain is known as to its constitution or origin, except that it is apparently not derived from the bile pigments. It has marked reducing properties.

Urobilin occurs in fresh normal urine as its chromogen, urobilinogen. This is converted into urobilin by acids or by the action of light and oxygen. The amount excreted is markedly increased in fevers, in diseases of the liver and bile passages, by destruction of the red corpuscles, especially in pernicious anaemia, and during the absorption of blood clots. In certain of these cases the urobilin itself is found in the urine, and can be identified by its characteristic absorption band, urobilinogen not giving a definite band.

Urobilinogen is a pyrrol body and is responsible for Ehrlich's reaction with p-dimethyl-amino-benzaldehyde.

The origin of urobilin from the bile pigments is discussed on page 119. It may be added that the urobilin absorbed from the bowel into the circulation is mostly excreted by the liver into the bile, so that only a small portion reaches the urine. Should the liver cells be injured there is a marked increase in the excretion of either urobilin or urobilinogen in the urine.

Uroerythrin is found in small amounts in normal urine. It is increased in fever and certain diseases of the liver.

It is soluble in amyl alcohol. Solutions have a reddish colour, but are unstable to light.

The pigment is usually associated with the urates or uric acid of the urine.

Haematoporphyrin is found in traces in normal urine. There is a certain increase in fevers, and some other diseases, but a very marked increase in certain cases of poisoning by sulphonal or trional, especially in women.

Urorosein occurs in urine as a chromogen which is converted into the pigment by the action of strong acids, such as HCl.

It is insoluble in ether and is thus distinguished from indigo blue formed in the test for indican. (Ex. 304.

The chromogen seems to be an indol body, possibly indol-acetic acid.

54. Note the colour of normal urine and examine some in a beaker by the spectroscope. Note that there are no definite absorption bands, but a general absorption of the violet. Urochrome, the chief urinary pigment, yields no bands.

55. Saturate at least 200 c.c. of urine with ammonium sulphate. Filter off the precipitate and let it dry completely in the air. Extract it with a small amount of strong alcohol. A brownish solution containing urobilinogen is obtained. Treat this with a few drops of hydrochloric acid: the urobilinogen is converted to urobilin. Examine with the spectroscope, and note a single absorption band situated at the junction of the blue and the green. Its centre is about λ 490.

D. The Inorganic Constituents.

Kations.

Sodium and potassium are found to the extent of 3·2 gm. K_2O and 5·23 gm. Na_2O per diem. The ratio $K_2O : Na_2O$ generally equals 1 : 1·54.

During starvation this can rise as high as 3 : 1, owing to the excretion of the potassium of the tissues, sodium being found in a much smaller amount than potassium. The same is found in all wasting diseases.

Calcium and magnesium are mainly excreted by the bowel. The amounts in urine are 0·33 to 0·6 gm. CaO and 0·16 to 0·24 gm. of MgO.

The amounts of these alkaline earths in the urine are increased by the administration of organic acids, or in conditions such as diabetes in which the formation of such acids is increased.

Iron also is mainly excreted by the bowel. It is found in human urine only in organic combination, and then only to the extent of 0·5 to 10 milligrams per diem.

Anions.

Chlorides form the chief part of the anions of the urine. The amount excreted is often calculated as if it all existed as NaCl, though the amount of sodium in the urine is normally not sufficient to combine with all the chlorine. The amount in the urine depends largely on the amount in the food, but since an important function of the kidney is to maintain a constant osmotic pressure of the tissue fluids, mainly by variations in the amount of NaCl excreted, it follows that anything tending to cause a change in the osmotic equilibrium in the body is liable to alter the excretion of chlorides in the urine.

Thus during starvation and during the formation of exudates in pneumonia the chlorides may disappear from urine. The amount of Cl excreted per diem is about 7 gms. Reckoned as NaCl it is 12 grams.

For the method of estimation see Ex. 318.

Sulphates. Only a small portion of the sulphate excreted in the urine is taken in as such with the food. The greater portion is derived from the oxidation of sulphur containing substances, chiefly proteins. The amount of sulphates is thus a rough measure of the total amount of protein metabolised, the ratio $\frac{N}{SO}$ being usually $\frac{5}{1}$

Sulphates are excreted very rapidly after a protein meal, reaching a maximum about the third hour. This seems to indicate that cystine, the sulphur complex of proteins, is split off and absorbed very early in the digestion of proteins.

Ethereal sulphates are esters formed by the union of sulphuric acid with phenols.

The proportion of the sulphur that is present as ethereal sulphate varies considerably. Folin has shewn that in starvation and on diets relatively deficient in proteins the proportion increases, as does that of the "neutral" sulphur. There is also a marked increase after the administration of certain phenolic substances, or when such compounds are formed in the body by bacterial decomposition, as in intestinal obstruction and severe constipation. In such cases the phenols found conjugated with sulphuric acid are

C_6H_5OH.....phenol

$C_6H_4\begin{matrix}(1)CH_3\\(4)OH\end{matrix}$.....p-cresol formed from tyrosine.

C_8H_6NOH.....indoxyl, formed from tryptophane.

These bodies are poisonous. They unite with sulphuric acid, probably in the liver, to form the innocuous ethereal sulphates.

The ethereal sulphates form soluble barium salts, and can be separated from the inorganic sulphates by treatment with barium chloride and filtering. They are hydrolysed to the phenol and sulphuric acid by boiling with hydrochloric acid.

"Neutral" Sulphur. In urine there is always present a certain amount of sulphur in a form less oxidised than that of a sulphate. The exact nature of the compounds in urine containing sulphur in this form is not yet clear.

It is probable that the amount of "neutral" sulphur in the urine is independent of the total amount of sulphur excreted. It probably varies with the amount of tissue protein metabolised, so that its determination is often of considerable interest.

For the percentages of sulphur excreted in the three forms under different metabolic conditions see page 123.

For the methods of determination of the sulphur see Exs. 320-322.

Phosphates. The phosphates of the urine are present on the one hand as salts of the alkali metals and of ammonium: on the other, as salts of the alkaline earths, calcium and magnesium. About 3·9 grms. of PO are excreted per diem in the urine. Phosphoric acid forms three series of salts. The formulae for that of sodium and calcium are

Normal phosphate, Na_3PO_4 : $Ca_3(PO_4)_2$.

Mono-hydrogen phosphate, Na_2HPO_4 : $CaHPO_4$.

Di-hydrogen phosphate, NaH_2PO_4 : $CaH_4(PO_4)_2$.

The three sodium salts and $CaH_4(PO_4)_2$ are soluble in water: the other two calcium salts are insoluble. The normal and mono-hydrogen phosphates are alkaline in reaction to litmus: the di-hydrogen phosphates are acid.

The phosphates of the urine are derived partly from the inorganic phosphates of the food, partly from the oxidation of phosphorus-containing substances of the food and tissues, such as nucleo-proteins, lecithins and phospho-proteins, and partly also from the phosphates of bone. The exact share played by these various compounds in forming the urinary phosphates is difficult to determine owing to the fact that a proportion of the phosphates varying between 12 and 50 per cent., are excreted by the

bowel. In this connection it may be noted that alkaline phosphates of the food are more likely to be excreted in the urine than are earthy phosphates.

The excretion of varying amounts of phosphates by the kidney is one of the methods by means of which the reaction of the body fluids is maintained in equilibrium. An increased excretion is always seen in cases of acid poisoning and in the acidosis associated with diabetes.

As soon as the urine shews a certain grade of alkalinity, a precipitation of earthy phosphates takes place. This is sometimes known as phosphaturia, but it is not necessarily associated with an increase of phosphates in the urine. In the phosphaturia of juveniles it is probable that there is an excessive amount of calcium in the urine, due to a defective excretion of the large intestine.

A certain amount of phosphorus is found in the urine in an organic form, not as a phosphate. It may be present as glycero-phosphoric acid. The average daily amount is about 50 mgms.

For method of estimation see Ex. 319.

6. Test for **chlorides** by adding to about 3 c.c. of urine a few drops of pure nitric acid and 3 c.c. of a 3 per cent. solution of silver nitrate. An abundant curdy precipitate of silver chloride appears at once. If the chlorides are less in quantity, the solution merely becomes milky or opalescent.

Note. If nitric acid is not added [illegible]

. To a test tube nearly full of urine add a little strong ammonia and boil. A white flaky precipitate of the **phosphates of calcium and magnesium** is formed. Filter off the precipitate, wash with water, and dissolve in 5 c.c. of dilute acetic acid. Divide the solution into two parts. To one part add a solution of potassium

oxalate. A white precipitate is produced, showing the presence of **calcium** in the urine.

[illegible]. To the other portion of the solution add an equal bulk of strong nitric acid and about 5 c.c. of ammonium molybdate. Boil: a yellow crystalline precipitate is produced, showing the presence of **phosphates.**

[illegible]

[illegible]. **To demonstrate the presence of acid-phosphates in urine.** Treat 5 c.c. of urine with an equal volume of 5 per cent. solution of barium chloride. Filter repeatedly through a small filter paper till the filtrate is clear. Treat the filtrate with a little baryta mixture and boil. Filter; dissolve the precipitate in [illegible] and add to the solution [illegible] precipitate. [illegible] acid phosphate, NaH_2PO_4.

[illegible]

[illegible]. [illegible] drops of [illegible] chloride solution and [illegible]. A precipitate of barium **sulphate** is produced as an opaque milkiness. [illegible] phosphates are [illegible]. Hydrochloric acid [illegible] prevent the precipitation of phosphates.

[illegible]. **To demonstrate the presence of ethereal sulphates.** To urine add an equal bulk of baryta mixture (two parts of baryta water [illegible] per cent. solution of barium nitrate). A precipitate [illegible] the precipitate and the filtrate *inorganic sulphates.* Filter till quite clear. To the filtrate add a

third of its volume of strong hydrochloric acid, boil in a beaker for two minutes, and allow to stand. A faint white cloud of barium sulphate is formed indicating the presence of *ethereal sulphates* in the urine.

Notes. 1. The ethereal sulphates form soluble barium salts but are [illegible]

$$C_6H_5 - O \qquad \qquad C_6H_5 - O$$
$$\Big\rangle SO_2 + H_2O = \Big\rangle SO_2 + H_2SO_4$$
$$HO \qquad \qquad HO$$

[illegible]

[illegible]

E. Urea.

Urea is the compound in which the greater part of the nitrogen is normally excreted in man. The percentage of the urinary nitrogen in the form of urea varies. Normally it is about 90 per cent., but in starvation, or on a diet deficient in proteins, it is only about 60 per cent. It is also low in cases of diabetes accompanied by acidosis (owing to the relatively high percentage of ammonia), and also in certain cases of hepatic disorder, notably acute yellow atrophy of the liver, owing to the non-formation of urea by the disordered liver, its seat of formation in the body.

The total amount excreted per diem by a normal man on an average diet containing 100 grams. of protein is 30 grams.

Urea is also known as carbamide, since it is the diamide of carbonic acid.

$$O{=}C{<}\begin{matrix}OH\\OH\end{matrix} \qquad\qquad O{=}C{<}\begin{matrix}NH_2\\NH_2\end{matrix}$$

Carbonic acid. Urea

Urea crystallises in water-free, colourless, long needles, or in four-sided prisms of the rhombic system which melt and decompose at 130 – 132 C.

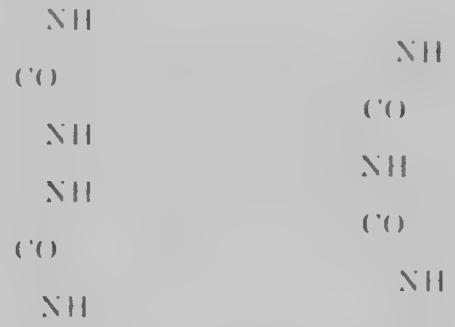
It is soluble in all proportions in hot water, and to the extent 1 : 1 in cold water. In cold alcohol it is soluble to the extent of 1 : 5. It is also soluble in acetone. Insoluble in pure ether and chloroform. The solutions are neutral in reaction.

It forms crystalline compounds with acids. The two most important are urea nitrate $CH_4N_2O.HNO_3$, insoluble in strong nitric acid, and urea oxalate $(CH_4N_2O)_2.C_2H_2O_4$, insoluble in oxalic acid.

It forms compounds with the salts of the heavy metals, especially with mercuric nitrate (see below, Ex. 250).

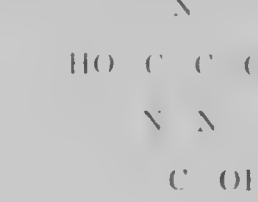
With reducing sugars relatively stable compounds are formed, called ureides. They are of importance in connection with the estimation of urea in diabetic urine.

On heating dry urea to 140 C., ammonia is evolved and biuret formed.



Biuret

On heating beyond 140 C., cyanuric acid and ammonia are formed. Cyanuric acid is $C_3H_3N_3O_3$.



Solutions of urea are decomposed by boiling alkalies into CO_2 and NH_3. They are also similarly decomposed by heating for several hours at 150° C. with acids. This decomposition is readily effected by the addition of magnesium chloride, zinc sulphate or potassium acetate to the solution for the purpose of raising the boiling point.

Bacteria, as micrococcus ureae, decompose urea into CO_2 and NH_3. This accounts for normal urine rapidly becoming ammoniacal on standing in the air.

Nitrous acid decomposes urea as follows:

$$CO(NH_2)_2 + 2HNO_2 = 2N_2 + CO_2 + 3H_2O.$$

Hypobromites effect a similar decomposition.

$$CO(NH_2)_2 + 3NaBrO = 3NaBr + CO_2 + N_2 + 2H_2O$$

Sodium hypobromite

242. To a watch-glass half full of distilled water add as much solid urea as will lie on a sixpenny-piece. Note the solubility of urea in water.

243. Place a drop of the urea solution on a slide, add a single drop of a saturated solution of oxalic acid, mix by stirring with a needle or fine glass rod, cover with a slip and examine the crystals of *oxalate of urea* that separate out. They vary considerably, containing long, thin, flat crystals, often in bundles and rhombic prisms. Draw the crystals.

244. Dilute the urea solution with twice its volume of water. Place a drop on a slide, add a drop of pure nitric acid, cover with a slip, and examine the crystals of *urea nitrate* that separate out. They form octahedral, lozenge-shaped, or hexagonal plates, often striated and imbricated. Draw the crystals.

245. Powder two or three crystals of urea in a watch-glass: rub with a small amount of acetone and warm gently on a water bath. The urea dissolves. Allow most of the acetone to evaporate away, and then place a drop of the remaining solution on a watch-

48. [illegible] Decomposition of urea by nitrous acid.

[illegible] Urea is decomposed by nitrous acid, carbonic acid and nitrogen being evolved.

[illegible] Urea is precipitated from its aqueous solution by [illegible] When a little nitric acid has been added to the solution, and then a solution of sodium nitrite, decomposition takes place.

$$CO(NH_2)_2 + HNO_2 = CO_2 + N_2 + 3H_2O.$$

Note. [illegible]

49. [illegible] To a solution of urea add sodium hypobromite. A mixed effervescence of nitrogen and carbonic acid gas takes place.

$$CO(NH_2)_2 + 3NaBrO + 2NaHO = 3NaBr + Na_2CO_3 + 3H_2O + N_2.$$

50. To a few c.c. of saturated ammonium sulphate add sodium hypobromite. A mixed effervescence of nitrogen and carbonic acid gas takes place.

$$(NH_4)_2SO_4 + 3NaBrO + 2NaHO = 3NaBr + Na_2SO_4 + 5H_2O + N_2.$$

Note. [illegible]

$$NaHO + Br_2 = NaBrO + NaBr + HO$$

[illegible]

$$3NaBrO = NaBrO_3 + 2NaBr$$

[illegible]

51. To some of the urea [illegible] add a solution of mercuric nitrate. A white precipitate of mercuric oxide combined with urea

[illegible]

Treat a solution of urea with Millon's reagent, and heat. A white precipitate is formed, owing to the presence of mercuric nitrate in the reagent. There is also an evolution of gas due to the action of the nitrous acid on the urea.

[illegible]. Boil 1 c.c. of a dilute solution of urea with a little strong alkali for fifteen minutes. Cool, neutralise with diluted sulphuric acid and test for urea by the addition of mercuric nitrate. No precipitate is obtained owing to the hydrolysis of the urea by the [illegible] alkali.

$$CO(NH_2)_2 + H_2O = CO_2 + 2NH_3$$

[illegible]. Place a little urea in a dry test tube. Heat carefully over a flame, keeping the upper part of the tube cool. The urea melts and evolves ammonia whilst a white sublimate condenses on the cooler parts of the tube. Cool the tube, add a little water and shake. Pour the solution into another tube and treat it with an equal bulk of sodium hydroxide and a drop of copper sulphate. A pink colour is produced, due to the biuret formed from the urea.

54. Repeat the experiment, but heat more strongly till the melt solidifies and becomes opaque. Cool, add two or three c.c. of water, boil and filter whilst still hot. Divide the solution into two portions A and B. To A add a few drops of a solution of

[illegible] acid and a [illegible] drop [illegible] coma. A white [illegible] crystallised [illegible].

[illegible] Add [illegible] to dissolve it and boil. On cooling an amethyst precipitate of copper ammonium cyanurate [illegible].

[illegible]

To demonstrate the presence of urea in urine. Treat 5 c.c. of urine with half its bulk of baryta mixture, and filter off the precipitate of sulphates and phosphates. Neutralise the filtrate with acetic acid and add a little mercuric nitrate. A white precipitate, soluble in sodium chloride, is obtained, indicating the presence of urea. (See Ex. 25.)

NOTE – [illegible]

Isolation of urea from urine. Evaporate about 30 c.c. of urine to complete dryness, finishing the evaporation on the water bath (to prevent the destruction of the urea). Turn out the flame and rub the residue with about 10 c.c. of acetone till it is boiling. Allow the acetone to boil, stirring all the time, till about half of it has evaporated away. Pour off the acetone into a dry watch glass and allow it to cool. Crystals of urea separate out as silky needles. Demonstrate that they are urea crystals by evaporating to dryness, taking up in a small amount of water and obtaining characteristic crystals of urea nitrate. (See Ex. 24.)

F. Uric Acid.

Uric Acid, $C_5H_4N_4O_3$, is 2-6-8-tri-oxy-purine.

$$\begin{array}{lll} NH-CO & & \\ CO \quad C-NH & & \\ & & CO \\ NH \quad C \quad NH & & \end{array}$$

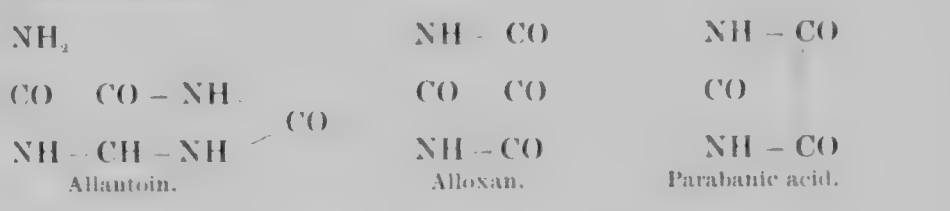
Its relationship to certain of the other purines is indicated on page 20.

When pure it crystallises in microscopic rhombic plates, but when impure it assumes a variety of forms, such as whetstones, dumb-bells, sheaves, rosettes, butchers' trays, etc.

It dissolves to the extent of 1 part in 16,000 parts of cold water and 1600 parts of hot water. It dissolves in alkalies, and the alkali salts of carbonic, phosphoric, boric, lactic and acetic acids, but not in the ammonium salts of these acids. It dissolves in warm concentrated sulphuric acid to form a sulphate, which is decomposed by the addition of water.

It is precipitated by phosphotungstic acid in the presence of hydrochloric acid, slowly by lead acetate, and completely by picric acid, mercuric chloride and ammoniacal silver nitrate.

By oxidation allantoin, alloxan, parabanic acid and urea are formed, depending on the reaction and the reagent employed.

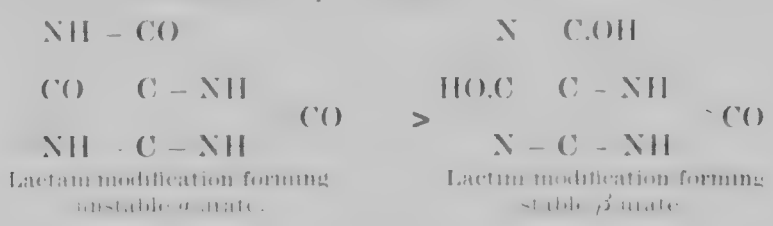


Allantoin. Alloxan. Parabanic acid.

Although the aqueous solutions of uric acid react neutral, it behaves like a disbasic acid $C_5H_2N_4O_3H_2$ and can form two series of salts, $C_5H_2N_4O_3Na_2$ (neutral, normal, or di-sodium urate) and $C_5H_2N_4O_3HNa$ (biurate, acid urate or mono-sodium urate). It is also possible that there is a third form of salt, $C_5H_2N_4O_3HNa.C_5H_3N_4O$ (quadriurate or hemi-sodium urate), though this may be merely a mixture of its two constituents. The di-sodium salts are more soluble than the mono-sodium, but are

only stable in markedly alkaline solutions. In the blood and urine urates exist as mono-sodium salts, which react neutral.

It is interesting to note that there are two modifications of the mono-sodium salt, called the α- and β-form. The α-form is more soluble than the β-form, but is unstable, and slowly passes over into the other form. They are probably the salts of the two tautomeric modifications of uric acid described by Fischer:



Lactam modification forming unstable α-urate.

Lactim modification forming stable β-urate.

It is of great interest to observe that in gout the amount of urate in solution in the blood is in excess of the amount of the β-urate that can be held by normal blood. So that in gout it must be present at least, partly, in the unstable α-form. The deposition of urates in the tissues during an acute attack may be due to the conversion of the unstable α- into the stable, less soluble β-modification.

Urates are completely precipitated as amorphous ammonium urate by saturation with ammonium chloride.

They exert a reducing reaction on Fehling's solution and towards alkaline silver solutions, this being the basis of Schiff's test.

They yield a characteristic colour reaction when evaporated with nitric acid, the so-called murexide test.

Uric acid occurs to the extent of about 0·7 gm. in the 24 hours' urine, but the amount excreted varies with the diet and the individual.

From its close chemical relationship to the purine bases formed by the hydrolysis of the nucleins of the food and tissues (see p. 20), the view is commonly held that uric acid has its origin in the cellular organs of the body from the oxidation of such substances. Thus we can have uric acid arising exogenously from the free or combined purines of the food and also endogenously from those of the tissues. This view is apparently supported by the fact that the administration of foods rich in nucleoproteins, as sweetbreads, or of certain of the pure purine bases, does cause an increased excretion of uric acid.

Plimmer has remarked on the close relationship between the elimination of uric acid and the number of leucocytes in the blood, and makes the suggestion that uric acid is a product of the metabolism of the leucocytes. This is not a revival of the old theory that it is formed by the disintegration of these cells.

It is important to note that a certain proportion of the uric acid formed in the body is destroyed by the liver, so that the amount excreted is a balance between that formed and that destroyed.

In gout, in which there is a deposition of uric acid in the tissues, the excretion is decreased before an acute attack, is increased during the attack, and then falls again. In this condition there is a recognisable amount of uric acid in the blood (see above). For methods of estimation in urine see Exs. 314, 315.

257. Treat a small amount of uric acid with 10 c.c. of 5 per cent. sodium carbonate. Heat nearly to boiling and cool. Note that a considerable portion of the uric acid has dissolved in the form of a urate.

258. Filter the solution and treat a portion with a drop or two of strong hydrochloric acid and shake. A white crystalline precipitate of uric acid separates out, showing that uric acid is very

insoluble in water. Allow the crystals to settle, remove a few by means of a pipette, and examine them microscopically. They usually form rhombic plates. Draw the crystals.

NOTE. If the solution is very strong the uric acid may separate out in an amorphous form. Should this be the case, make the solution alkaline and heat to dissolve. Whilst still hot add some HCl and allow the tube to cool slowly.

Uric acid can assume a great variety of crystalline forms resembling dumb-bells, whetstones, butcher trays, stars, and sheaves.

259. To another portion of the solution add two drops of ammonia and saturate with ammonium chloride. A white amorphous precipitate of ammonium urate is formed.

NOTE. This is the basis of Haycraft's method for the estimation of urates in urine. It is an important reaction for separating urates from physiological fluids, such as urine (see Ex. 268), since no other organic substance, likely to be met with in physiological analysis, is precipitated by saturation with ammonium chloride. The murexide reaction can be applied to the precipitate obtained.

260. Treat a little uric acid with a little strong sulphuric acid: it dissolves. Pour the solution into water: the uric acid may separate out.

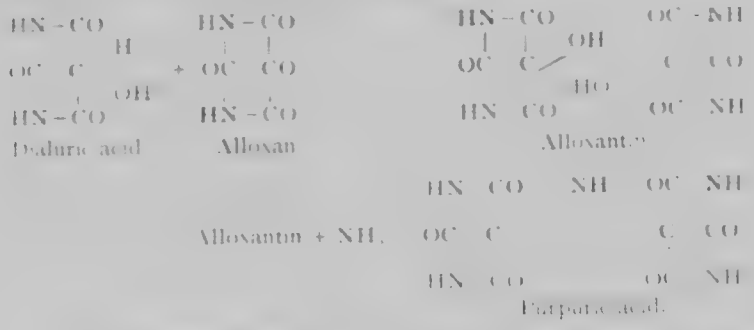
261. **Murexide test.** Treat a little uric acid in a porcelain dish with two or three drops of strong nitric acid. Heat on the water-bath till every trace of nitric acid and water has been removed. A reddish deposit remains. Treat this with a dilute solution of ammonia (five drops of ammonia to about a test tube full of water). The residue turns reddish violet in colour. Add a little caustic soda. The colour turns to a blue-violet.

NOTE. 1. This important test needs a certain amount of care. The heating must be performed on the water-bath and should be continued as long as is necessary to ensure the complete removal of every trace of nitric acid.

2. Xanthine and guanine give a yellow substance (nitro-xanthine) when treated with nitric acid. On evaporation the colour goes to a violet shade, which turns yellow with dilute ammonia. Adenine and hypoxanthine give no colour reaction.

3. The chemistry of the reaction is as follows: From uric acid arises by oxidation dialuric acid and alloxan. They condense together to form

...antin. By the action of ammonia on alloxantin, purpuric acid ... Murexide is ammonium purpurate



Dialuric acid Alloxan Alloxantin

Alloxantin + NH_3

Purpuric acid.

262. **Schiff's test.** Treat a very small amount of uric acid with a few c.c. of sodium carbonate. Pour the solution on to filter paper moistened with silver nitrate. A black stain of reduced silver immediately results.

NOTE.—This useful test cannot be applied in the presence of chlorides. It is important to note that the uric acid is dissolved in sodium carbonate, not the hydroxide, as the latter gives a precipitate of the brown silver hydroxide, which completely obscures the reduction. An amount of sodium carbonate in excess of that required to dissolve the uric acid must be added, as the reduction only takes place in the alkaline condition.

263. **Folin's test.** To a *very* small pinch of uric acid in a beaker add 20 c.c. of a saturated solution of sodium carbonate. Stir till the uric acid has completely dissolved, add 1 c.c. of Folin's uric acid reagent. A blue colour is obtained.

NOTES. **Preparation of Folin's solution.** 100 grams of pure sodium tungstate, 102 c.c. of pure ortho-phosphoric acid (B.P. 66·3%) and 750 c.c. of distilled water in a flask fitted with a reflux condenser are boiled for 2 hours. On cooling the solution is diluted to 1 litre.

2. The solution also gives a blue colour with polyphenols. It is used for the microchemical estimation of uric acid in urine (Ex. 315).

264. Dissolve a little uric acid in sodium carbonate by boiling. Add 5 c.c. of Fehling's solution and boil for a considerable time. Note the peculiar reduction of the copper, and compare it with the reduction obtained with glucose.

265. Similarly try the effect of uric acid on Nylander's (Ex. 70) and Benedict's (Ex. 68) solutions. A reduction is not obtained.

266. Dissolve some uric acid in sodium carbonate, add an excess of ammonia and treat with silver nitrate. A white amorphous precipitate of a silver compound of uric acid is formed.

[illegible]

267. **A solution of sodium urate and urea is provided. To prepare crystals of uric acid and of urea.**

Heat a test tube nearly full of the solution to boiling point and add [illegible] acetic acid. Allow the tube to cool [illegible] uric acid crystals separate out. Cool [illegible] Filter off the uric acid. Neutralise the filtrate with sodium carbonate and evaporate to dryness, finishing the process on the water-bath, to prevent the conversion of the urea into [illegible]. (See Ex. 253.) Extract the residue with strong alcohol or acetone. The alcohol or acetone solution is carefully evaporated to dryness, and the urea crystallises out.

268. **To demonstrate the presence of uric acid in urine.**

To [illegible] c.c. of urine with two drops of ammonia and then stir with powdered ammonium chloride till the solution is saturated. Allow the excess of ammonium chloride to settle for 15 secs., and pour off into another beaker. Note the gelatinous precipitate of ammonium urate. Filter: scrape the precipitate off the paper and transfer it to an evaporating dish. Add three or four drops of strong nitric acid and place the dish on the water bath till a pink, dry residue is obtained. Treat this with a little dilute ammonia: the purple colour produced indicates the presence of urates in urine. (See Exs. 259 and 261.

269. **Folin's method of demonstrating the presence of uric acid in urine.** To 1 to 2 c.c. (20 drops) of urine in an evaporating dish add one drop of a saturated solution of oxalic acid and evaporate to *complete* dryness on a water bath. Allow to cool, add 10 c.c. of strong alcohol and allow to stand for five minutes to extract the polyphenols. Carefully pour off the alcohol. To the residue add 10 c.c. of water and a drop or two of saturated sodium

...bonate. Stir to secure complete solution of the uric acid and transfer to a beaker. Add 1 c.c. of Folin's uric acid reagent (Ex. ...) and 20 c.c. of saturated sodium carbonate solution. The blue ...lour that results indicates the presence of uric acid.

70. Urine has been treated with about one-fiftieth its bulk ...strong hydrochloric acid, and allowed to stand from twelve to ...twenty four hours. Note the brown crystals of uric acid that have ...ttled on the sides of the vessel. Examine them microscopically: ...they form very irregular crystals, usually arranged in sheaves. Draw the crystals.

NOTE.—The chief pigment that associates itself with uric acid and urates is ... see p. 143.)

G. Purine bases, other than uric acid.

The most important of these found in normal urine are hypoxanthine, xanthine and adenine (see p. 20), derived from the metabolism of food and tissue nucleins: heteroxanthine (7-methyl-xanthine) and paraxanthine (1, 7-dimethyl-xanthine) derived from the breakdown of caffeine (1, 3, 7-trimethyl-xanthine) and theobromine (3, 7-dimethyl-xanthine) of the coffee, tea and cocoa ingested.

In man the methylated xanthines constitute the greater part of these purine bases. But it is interesting to note that the non-methylated ones are much increased in fever. Also during severe muscular exercise there is an increase, accompanied by a decrease of uric acid. After the exercise there is an increase of uric acid, and a decrease of the other purines.

The simplest method of estimation is to determine uric acid nitrogen by the method in Exs. 314, 315, and the total purine nitrogen by applying Kjeldahl's method (Ex. 306) to the total purines precipitated by ammoniacal silver nitrate (Ex. 266). The difference is the nitrogen of the purine bases.

H. Creatinine and Creatine.

The chemical relationships of these bodies are described on p. 77. In normal human urine creatinine is always present, but creatine only after a meat diet, being derived from that of the food. Creatine, however, is a normal constituent of the urine of children.

Creatinine seems to be a product of tissue metabolism, and the amount excreted is regarded by Folin as a measure of endogenous metabolism. (See tables B and C, p. 123.) There is an increase in complete starvation and in fevers, due to the increased breakdown of the tissues. Mellanby has drawn attention to the fact that the liver is probably the seat of formation of creatinine. Thus in most diseases of the liver there is a decreased excretion, an important exception being hepatic carcinoma, in which condition the urinary-creatinine is increased and is accompanied by creatine. Creatine is excreted when the muscles of the body are broken down. This explains the presence of creatine in urine during starvation and in fevers.

When creatinine is given by the mouth it is mainly excreted unchanged, but a small portion is broken down into unknown products. When creatine is administered it also is chiefly excreted unchanged, but a certain percentage is destroyed in the body. The amount excreted unchanged is considerably increased with diets rich in proteins.

Properties. Creatinine dissolves in 11 parts of water and 102 parts of alcohol at 16 C. It is insoluble in ether. Its solutions are neutral or very slightly alkaline in reaction.

Creatinine is precipitated by phosphotungstic acid, by picric acid, and by the salts of the heavy metals.

Alkalies convert it slowly into creatine. On boiling with barium hydroxide it is converted into urea and sarcosine (see p. 77).

Creatinine reduces Fehling's solution, but not Benedict's or Nylander's solutions.

For the method of estimation see Ex. 316.

271. **Jaffé's test.** To 5 c.c. of urine add a few drops of a saturated aqueous solution of picric acid and of a 10 per cent. solution of sodium hydroxide. A red colouration is produced owing to the formation of picramic acid.

272. **Weyl's test.** To 5 c.c. of urine add a few drops of a freshly prepared 5 per cent. solution of sodium nitroprusside. Add a 5 per cent. solution of sodium hydroxide, drop by drop. A ruby-red colour appears, which quickly turns yellow.

NOTE.—Acetone gives a similar red colour, but it does not turn yellow.

273. **Salkowski's test.** To the yellow solution obtained in the preceding exercise add an excess of acetic acid and boil. A greenish blue colour results. On standing, a sediment of Prussian blue may separate.

I. Ammonia.

Ammonia is a constituent of normal urine, being present to the extent of about 0·7 gm. per diem. There is an increased excretion following the administration of ammonium salts of inorganic acids, in certain cases of hepatic disease, and as a result of acid poisoning. This last condition ("acidosis") can be produced by the administration of inorganic acids or by the excessive formation of acids in the body, especially if this is not accompanied by an increased intake of alkalies. Thus it is seen in severe diabetes, in starvation, and in delayed chloroform poisoning, the acids formed being aceto-acetic and β-oxy-butyric acids.

For methods of estimation see Exs. 308 to 310.

J. Hippuric Acid.

Hippuric acid is formed in the kidney by the condensation of benzoic acid with glycine.

$$\underset{\text{Benzoic acid.}}{C_6H_5COOH} + \underset{\text{Glycine.}}{H_2NCH_2COOH} = \underset{\text{Hippuric acid.}}{C_6H_5CONHCH_2COOH} + H_2O$$

The amount excreted by a normal individual on a mixed diet is about ·7 gm. per diem. It is increased by a vegetable diet, owing to the presence in most plant foods of an aromatic complex that is oxidised to benzoic acid in the body.

Hippuric acid crystallises in 4-sided prisms, somewhat resembling triple phosphate. It melts at 187·5 C.: above this temperature the melt becomes red and is decomposed into benzoic acid, benzonitrile and prussic acid. It is soluble in hot water, alcohol and ethyl acetate: insoluble in benzene and petroleum ether: only slightly soluble in cold water, alcohol, ether and chloroform. It forms an insoluble ferric salt. By hot acids or alkalies it is hydrolysed to benzoic acid and glycine. When evaporated with strong nitric acid, nitrobenzene is formed.

74. **Isolation from urine by Roaf's method.** 500 c.c. of the urine of a horse or cow are treated with 125 grams of ammonium sulphate and 7·5 c.c. of concentrated sulphuric acid. On standing for 24 hours the hippuric acid crystallises out. Filter off the crystals, and wash with a little cold water. Dissolve in a small amount of hot water, boil with a little animal charcoal, filter, concentrate if necessary, and allow to stand for 24 hours.

75. To a little hippuric acid in a small evaporating dish add 1·5–2 c.c. of concentrated nitric acid and evaporate to dryness in a water-bath in the fume chamber. Transfer the residue to a dry test tube, apply heat, and note the odour of nitrobenzene (artificial oil of bitter almonds).

276. Neutralise a portion of the urine with dilute caustic soda. Add a few drops of ferric chloride. A cream-coloured precipitate of the ferric salt of the uric acid is formed.

K. Certain Constituents of Abnormal Urine.

1. Albumin and Globulin.

"Albuminuria" is the name given to the condition in which a heat-coagulable protein is found in the urine, no matter whether the protein present is albumin or globulin. As a rule both proteins are present, but albumin is generally greatly in excess of the globulin.

Albuminuria can be renal ("true") or accidental ("false"). Renal albuminuria can be brought about by an alteration in the blood pressure in the kidney, by a change in the composition of the blood, or by an alteration in the structure of the kidney. In accidental albuminuria, the protein is not passed by the kidney, but gains access to it lower down in the urinary tract. It is generally accompanied by haemoglobinuria.

For the method of estimating the albumin see Exs. 323, 324.

277. **Boiling test.** Filter the urine till it is clear. If it will not filter clear, as when infected with bacteria, shake with kieselguhr and filter again. If the urine be alkaline to litmus, make it faintly acid by the cautious addition of 1 per cent. acetic acid. Fill a narrow test tube three parts full with the clear urine, incline it at an angle and boil the upper layer by means of a very small flame. A turbidity indicates either albumin or earthy phosphates (see note 2 to Ex. 9). Add one or two drops of strong acetic acid, boiling after the addition of each drop. Any remaining turbidity indicates the presence of albumin.

278. **Heller's test.** Place about 3 c.c. of pure nitric acid in a narrow test tube. Float about 3 c.c. of filtered urine on the surface of this, using a pipette to avoid mixing. A white ring at the junction of the fluids indicates the presence of albumin.

Notes. 1. The precipitate is due to the formation of metaprotein by the action of the acid on the albumin, and the insolubility of the metaprotein in the excess of nitric acid. (See Exs. I. 14 and 15.)

2. A coloured ring is usually produced owing to the oxidation of certain chromogens.

3. If the urine is very rich in albumin, the precipitate usually has very sharply defined limits.

4. If the urine is very rich in urates, a precipitate of uric acid may form at the junction of the fluids, or more commonly, somewhat above the surface. Urea and uric acid are distinguished from albumin by the precipitation of the urine with two or three volumes of alcohol.

5. The presence of resinous substances in the urine of patients who have been treated with balsams leads to the development of a white ring of resin that disappears on treatment with alcohol.

6. Urine rich in albumoses may give a white cloud that disappears on warming.

7. Urine that has been preserved by the addition of thymol gives a ring of nitrosothymol or nitrothymol. The thymol can be removed by gentle agitation with petroleum ether.

279. **Roberts' test.** Repeat the previous exercise, using Roberts' reagent in place of the nitric acid. A white ring at the junction of the fluids indicates albumin.

Notes. 1. Roberts' reagent is prepared by adding 1 volume of pure nitric acid to 5 volumes of a saturated solution of magnesium sulphate.

2. Coloured rings are not formed, and so confusion is avoided.

280. **Spiegler's test.** Render the urine faintly acid with acetic acid and repeat the above test, using Spiegler's reagent in place of Roberts'. A white ring indicates the presence of albumin.

Notes. 1. Spiegler's reagent consists of

Mercuric chloride	40 gm.
Tartaric acid	20 gm.
Glycerine	100 gm.
Sodium chloride	50 gm.
Distilled water	1000 c.c.

2. The reaction is also given by albumoses and peptones.

3. The test serves to show 1 part of albumin in 250,000. It is almost too delicate for ordinary clinical work, as a large number of apparently normal urines give a positive reaction.

2. *Albumoses.*

Albumoses are found in the urine in certain cases of degeneration of the intestinal epithelium ("alimentary albumosuria"). Also in a variety of other conditions such

as in the absorption of pneumonic exudates, in some cases of an increased breakdown of the tissues in certain fevers, in the puerperium, and in urine containing semen.

The albumose present seems to be a secondary albumose.

281. Remove any albumin that may be present by heat coagulation. To the filtrate apply Spiegler's test (Ex. 280). A white ring indicates the presence of albomose.

3. *Bence-Jones' Protein.*

In certain cases of disease of the bone marrow (multiple myeloma), and possibly in osteomalacia, a protein with peculiar properties is found in the urine. It is named after Bence-Jones, who first described the condition. It has the property of coagulating at temperatures under 55° C., of redissolving to a clear solution on boiling and of reappearing on cooling. It is precipitated by half-saturation with ammonium sulphate. It is not precipitated on dialysis.

Hopkins has shewn that the solution of the heat coagulum on boiling depends on the presence of neutral salts, those with divalent cations (as $CaCl_2$) being most potent in neutral or faintly acid solutions, and those with divalent anions (as K_2SO_4) in faintly alkaline solutions.

Hopkins has also shewn that the protein excreted is formed in the body, either in the marrow or as a result of the influence of the growth on general metabolism. The amount in the urine is independent of the nature or amount of the proteins of the food. The nitrogen of the protein excreted may be as high as one-third of the total urinary nitrogen.

282. If necessary make the suspected urine faintly acid with acetic acid. Heat carefully by immersing in a beaker of warm

water. The urine becomes turbid at 40° to 45° C., and shows a flocculent precipitate at 60° C. On raising the temperature to 100° C., the precipitate partially or completely disappears. On cooling it reappears.

4. Blood Pigments.

Blood pigments may occur in pathological urine in intact corpuscles ("haematuria") or free in solution ("haemoglobinuria").

Haematuria can be recognised by determining the presence of red corpuscles by a microscopic examination of the sediment obtained by centrifugalising the urine. It occurs with gross lesions of the kidney or any part of the urinary tract, so that blood passes directly into the urine. If the blood comes from the kidney it is well mixed with the urine. If the blood comes from the bladder or genital organs it often forms a clot. In haematuria the urine often has a characteristic smoky appearance, and it is always associated with albuminuria. Haemoglobinuria is a result of haemolysis. It therefore follows a variety of infectious diseases, transfusion of blood, the absorption of haemolytic substances, such as many aromatic compounds, severe burns and scalds. Methaemoglobin is nearly always present.

[illegible] **Heller's test.** Boil 10 c.c. of urine with a little 40 per cent. [illegible] solution, and allow the tube to stand for a while. A [illegible] indicates [illegible] per cent. of blood pigment in the urine. [illegible] Add to the [illegible] of blood and acidify with acetic acid. The [illegible] disappears, leaving a red colour.

[illegible]

[illegible]

Schumm's spectroscopic test. [illegible] c.c. of the [illegible] with [illegible] c.c. of acetic acid and 50 c.c. of ether. Shake

thoroughly in a separating funnel. Allow to stand and add a drop or two of alcohol to obtain a separation of the layers. Run off the urinary layer. To the ether add 5 c.c. of water, shake and run off the water. To the washed ether add ammonia and shake for half a minute, cooling under the tap. The reaction must be markedly alkaline after shaking. Run off the lower coloured layer into a tube, add 5 to 10 drops of ammonium sulphide solution and examine spectroscopically for the bands of haemochromogen. (Ex. 219.)

258. **Adler's benzidine test.** To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal bulk of 3 per cent. hydrogen peroxide and 1 c.c. of the urine. If the mixture is not acid, render it so by the addition of acetic acid. The appearance of a green or blue colour indicates the presence of blood pigment.

NOTES.—1. A control test should be performed, substituting water for urine.

2. The reaction can be applied to the acid ethereal solution prepared in preceding exercise.

3. Benzidine preparations vary considerably in sensitiveness. The directions must be kept in the dark.

5. *Bile.*

The constituents of the bile are found in urine when the bile duct is obstructed by a calculus or by catarrh. The bile is absorbed into the lymphatics, passes into the circulation and reaches all parts of the body, the pigments causing a staining of the various tissues. The condition is known as jaundice.

The absence of bile salts from the urine does not exclude the possibility of the presence of bile pigments. With continued obstruction of the bile passages the formation of bile salts seems to decrease. Urine containing bile often has a characteristic appearance.

259. **Cole's test for bile pigments.** To 25 c.c. of urine add Maryta mixture and proceed as directed in Ex. 227.

287. **Hay's test for bile salts.** Sprinkle the surface of some urine in a test tube with flowers of sulphur. The particles fall to the bottom of the tube if bile salts are present. (See Ex. 224.)

288. **Oliver's test for bile salts.** Acidify the urine with acetic acid and filter if necessary. To it add a clear 1 per cent. solution of Witte's peptone, also acidified with acetic acid. A white precipitate indicates bile salts. (Ex. [illegible].)

289. **Jolle's test for bile salts.** Treat 50 c.c. of urine with 15 c.c. of a 3 per cent. solution of casein, add 10 per cent. sulphuric acid, drop by drop, with continued stirring until the casein is completely precipitated (6 to 8 c.c. usually required). Filter, and treat the precipitate in a small beaker with 10 c.c. of strong alcohol. Allow to stand for 1 hour at room temperature, stirring frequently. Filter and treat 5 c.c. of the filtrate with one drop of a 5 per cent. solution of rhamnose and 5 c.c. of concentrated hydrochloric acid. Boil over a small flame, and keep gently boiling for about two minutes. Cool, add 2 c.c. of ether and shake. A characteristic green fluorescence indicates the presence of bile salts.

6. *Glucose.*

Glucose seems to be a constituent of normal urine, but the amount present is very small (0·01 to 0·04 per cent.). When present in recognisable quantities the condition is known as glycosuria.

There are two types of glycosuria, alimentary and persistent. Alimentary glycosuria is the condition in which the amount of sugar absorbed exceeds the amount that the individual is capable of assimilating. The limit varies with the individual, and is affected by a variety of pathological conditions. Persistent glycosuria is the condition when large amounts of sugar are excreted for a considerable length of time, and may be quite independent

of the administration of carbohydrate food. The condition is known as diabetes mellitus. The urine is generally much increased in amount, of a high specific gravity, and pale in colour.

Nylander's test. Ex. 70 is also valuable. The reagent is not reduced creatinine or uric acid. But certain substances of unknown composition that are occasionally found in urine cause a slight reduction. It [illegible] in a clinical sense. But should a positive [illegible] be due to some other substance.

The osazone test serves to confirm the [illegible] and especially to distinguish between [illegible] on the other. The fermentation test is also valuable especially in connection with the recognition of lactose and [illegible]

If proteins are present they must be [illegible] solution before performing the [illegible]

1. **Benedict's test.** To [illegible] for two minutes and allow to cool spontaneously. [illegible]

2. **Nylander's test.** [illegible] Ex. 70 in a [illegible] Boil and [illegible]

Fehling's test.

Phenylhydrazine test.

Cipollina's test.

Fermentation test.

Fructose.

Fructose occasionally occurs in the urine, sometimes being accompanied by glucose. The significance of fructosuria is not yet clear.

Seliwanoff's test

Pentose.

Pentoses, that is carbohydrates with 5 carbon atoms, appear in the urine in three conditions, alimentary, persistent or true pentosuria, and admixed with glucose in cases of glycosuria.

Alimentary pentosuria is sometimes seen after the ingestion of considerable quantities of certain fruits, as

prunes, cherries, grapes and plums. The sugar found varies, but is usually *d*-arabinose. In true pentosuria it is *dl*-arabinose. Its origin and significance have not yet been clearly established.

The presence of pentoses in urine is indicated when Nylander's reaction gives a grey and not a black precipitate: when Fehling's test shows a very slow reduction that often occurs quite suddenly as the mixture cools, and when the fermentation test is negative. The two colour reactions described are also given by glycuronic acid, which can, however, be demonstrated by Ex. 303.

297. **Tollen's test.** T

and between D and E

ont.

298. **Bial's orcin test.** To 2–3 c.c. of urine add 4–5 c.c. of

and the other near the D

9. *Lactose.*

Lactose is found in the urine of women during pregnancy, during the nursing period, and soon after weaning. The amount in the urine varies, but rarely exceeds 1 per cent. The excretion usually reaches its maximum 2 to 4 days after parturition.

It is not easy to demonstrate the presence of lactose in urine very satisfactorily. Barfoed's test is not applicable, owing to the fact that the reagent is reduced by the constituents of normal urine.

The osazone cannot be isolated with any certainty, owing to its solubility. Should a marked reduction occur, and if osazone crystals cannot be obtained, the fermentation test should be applied, using pure yeast that has been tested against lactose. If this be negative, then the sugar present is either lactose or a pentose. Should the tests for pentoses yield negative results, lactose is indicated. Its presence can be confirmed by obtaining crystals of mucic acid, which is yielded only by lactose or galactose.

(c) **Mucic acid test.** 100 c.c. of the urine and 20 c.c. of pure concentrated nitric acid are evaporated in a wide and rather deep beaker on a boiling water bath in a fume chamber. The evaporation is continued until the fluid becomes clear, and brown fumes are no longer evolved. The total volume is then about 10 c.c. Remove the beaker from the bath and transfer the contents to a smaller beaker, washing out with a small amount of distilled water. Allow to stand overnight in a cool place. The formation of a white crystalline mass of mucic acid indicates the presence of lactose in the urine. Dilute the fluid, collect the crystals on a small filter and wash with cold water. Microscopically the crystals are seen to be very pointed prisms at oblique angles. The melting point is 213–215° C. It can be weighed and titrated with standard alkalies, its equivalent being 105.

NOTE.—Mucic acid is $COOH(CHOH)_4COOH$.

10. The Acetone bodies.

The acetone bodies found in urine in the condition known as "acidosis" are

Acetone. $CH_3.CO.CH_3$.

Aceto-acetic acid. $CH_3.CO.CH_2.COOH$.

β-oxy-butyric acid $CH_3.CHOH.CH_2.COOH$.

β-oxy-butyric acid is readily oxidised to aceto-acetic acid, and this is converted into acetone by the loss of CO_2.

The two acids are never found in urine unaccompanied by acetone: but acetone may be present without the acids. The excretion of the acetone bodies depends on the inability of the tissues to oxidise completely the fatty acids generally derived from the fats, but sometimes from certain of the amino-acids formed in the metabolism of proteins. The condition that usually gives rise to acetonuria or acidosis is the inability of the tissues to obtain or to utilise an adequate amount of glucose. Thus these acetone bodies are excreted in starvation, on a diet of fats with a limited amount of protein, in certain fevers, severe anaemias, and after phosphorus poisoning, and finally in diabetes mellitus, in which condition the tissues are unable to utilise the glucose provided.

Rothera's test for acetone.

Gunning's iodoform test for acetone.

Gerhardt's test for aceto-acetic acid.

11. Glycuronic Acid.

Glycuronic acid, CHO. CHOH $_4$COOH, is not found free in the urine. It is found conjugated with certain drugs, or with substances formed from these in the body. These conjugated glycuronates are excreted after administration of chloral, camphor, naphthol, menthol, phenol, morphine, oil of turpentine, antipyrin, etc. The free and conjugated acids are reducing substances, but are not fermentable. They give the reactions for the pentoses, but can be distinguished by the test given below.

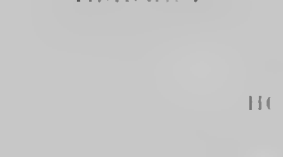
Tollen's test for glycuronates.

12. *Indican.*

Indican is the potassium salt of indoxyl sulphuric acid, and is thus one of the ethereal sulphates (see p. 135).

Indoxyl is

Indican is



Indoxyl arises from the bacterial decomposition of tryptophane in the intestine, thus differing from the other ethereal sulphates which are normal tissue metabolites (see p. 135). The excretion of indican is of importance as a measure of the amount of putrefaction occurring, generally in the intestine, but sometimes in a large abscess.

1. **Jaffé's test.** Treat 5 c.c. of urine with a rather large [illegible] concentrated hydrochloric acid and about 2 c.c. of [illegible]. Add a single drop of 3 per cent. potassium chlorate and shake. Allow the chloroform to settle and examine its colour. If it be blue, indican is present. If not, add another drop of the [illegible] and shake again. [illegible] blue colour is extended in the [illegible] absent.

Lavelle's test.

L. Urinary Sediments.

For the proper examination of these substances a hand centrifuge is desirable. The sediment obtained should be examined microscopically, and chemically if necessary.

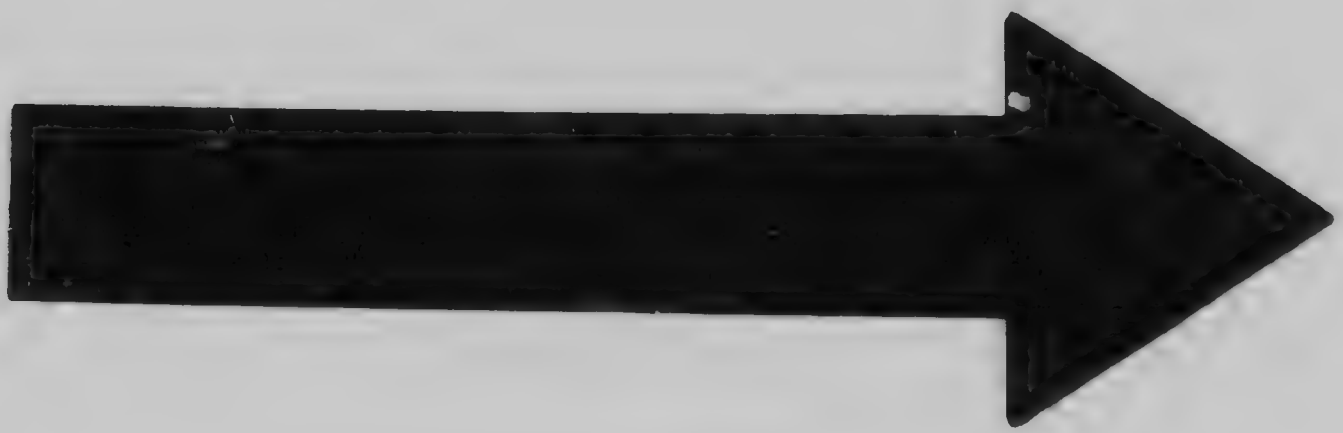
The sediments obtained are either organised or unorganised. Organised sediments consist of casts of the renal tubules, epithelial cells from different parts of the urinary tract, pus, blood cells, spermatozoa, parasites, etc. It is not thought advantageous to describe them in this book.

Unorganised sediments vary with the reaction of the urine. The more common varieties are given below.

In acid urine.

Uric acid: light yellow to dark reddish-brown in colour. Crystalline form very varied: rhombic prisms, wedges, rosettes, dumb-bells, whetstones, butcher's trays, etc. Soluble in sodium hydroxide and reprecipitated by hydrochloric acid.

Urates: pinkish, soluble on warming, sometimes amorphous, sometimes crystalline, as "thorn-apples," fan shaped clusters of prismatic needles.



Calcium oxalate: octahedra, with an envelope-like appearance (squares crossed by two diagonals); also in dumb-bells. Insoluble in acetic acid, easily soluble in hydrochloric acid.

Calcium hydrogen phosphates (stellar phosphates); in rosettes of prisms and in dumb-bells. Rather rare.

Cystine: colourless hexagonal plates, soluble in ammonia, insoluble in acetic acid. Very rare.

In alkaline urine.

Ammonium magnesium phosphate (triple phosphate): colourless prisms ("coffin-lids" and "knife-rests") or feathery stars. Easily soluble in acetic acid.

Alkaline earthy phosphates of calcium and magnesium: amorphous. Insoluble on warming and in alkalies, soluble in acetic acid.

Calcium hydrogen phosphate: see above.

Calcium carbonate: dumb-bells or spheres with radiating structure

Ammonium urate: yellow, or brownish amorphous masses, or shewing "thorn-apple" crystals. Soluble on warming.

CHAPTER X.

THE QUANTITATIVE ANALYSIS OF URINE.

To determine the nature of the metabolic processes in the body a sample of the measured 24 hours' urine must be analysed. In taking the 24 hours' urine it is best to finish with that voided after the night's rest. The total collected during the 24 hours is mixed and carefully measured. The analyses should be performed as soon as possible, owing to the risk of bacterial decomposition of certain of the constituents. Should it be necessary to postpone the analyses an antiseptic should be added. Toluol or thymol are the best to use (but see Ex. 278, note 7). Chloroform must not be used in any case, since it is decomposed by alkalies and has a marked effect on certain processes.

The analyses performed will vary with the nature of the case that is being investigated, and the time and apparatus at the disposal of the analyst. It is of the utmost importance for the student to acquire skill in the conduction of a complete analysis, and in this connection particular attention is directed to Folin's micro-chemical methods, based on colorimetric comparison, that are described below. They enable a complete analysis of the nitrogenous constituents of a sample of urine to be made in a few hours with a very small amount of special apparatus beyond a good suction pump and a reliable colorimeter, preferably Duboscq's

Since the fumes arising from the incineration of urine by boiling sulphuric acid are extremely irritating, that operation should be conducted in a fume chamber or under a hood. But these can be dispensed with by use of the special fume-absorber devised by Folin and illustrated in Fig. 6. A is a bulb 1½ inches in diameter blown into a piece of 5ths Jena tubing. The lower

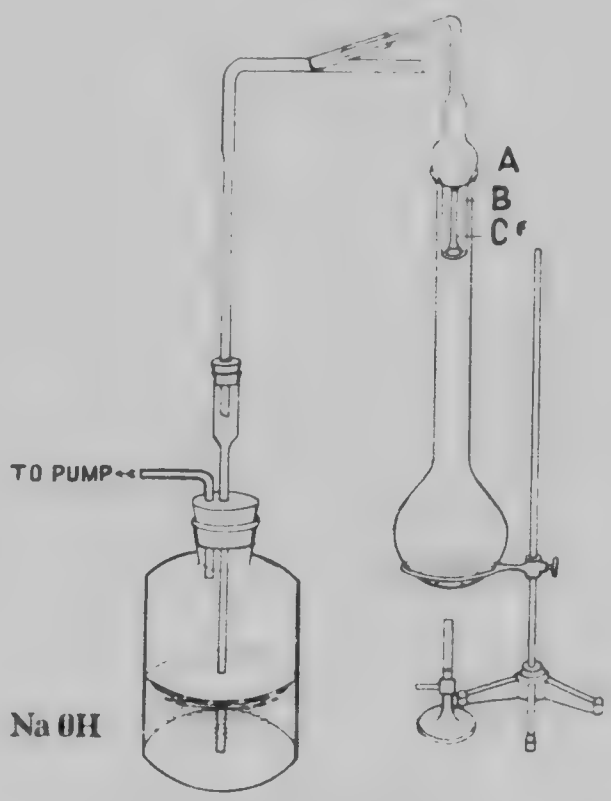


Fig. 6.—Folin's fume absorber.

* The apparatus described in this book can now be obtained from Messrs. J. Griffin and Sons, or from Messrs. Baird and Tatlock, and will be listed in their next catalogues.

and has blown into it a piece of narrow tubing (C) 2 inches in length. The bulb rests on the neck of the flask or test-tube in which the incineration is conducted.

To the upper end of the tube is fixed a piece of narrow tubing which is bent at a convenient angle, and which slips into a slightly longer tube connected to a good suction pump. The fumes are carried over by the air current into the pump, a wash bottle containing caustic soda being interposed to prevent damage. The condensation water collects in the pocket C and can be removed by inverting the fume-absorber at the end of the experiment. The removal of this condensation water materially hastens the incineration.

One good pump suffices to carry off the fumes from three or four incinerations simultaneously.

By inverting a funnel over an evaporating basin, and arranging the apparatus so that the end of the funnel fits loosely into the neck of the absorber, the fumes from boiling nitric acid can be carried off.

[illegible] The estimation of total nitrogen by Kjeldahl's method.

Principle. The nitrogen [illegible] converted into ammonia [illegible] acid, copper sulphate being added to aid the oxidation, and potassium sulphate to raise the boiling point. The mixture is diluted with water, made alkaline by [illegible] and the ammonia distilled into a measured amount of standard acid. The amount of this neutralised by the ammonia is found by subsequent titration with standard alkali. Knowing the amount of ammonia formed from 5 c.c. of urine, the percentage of

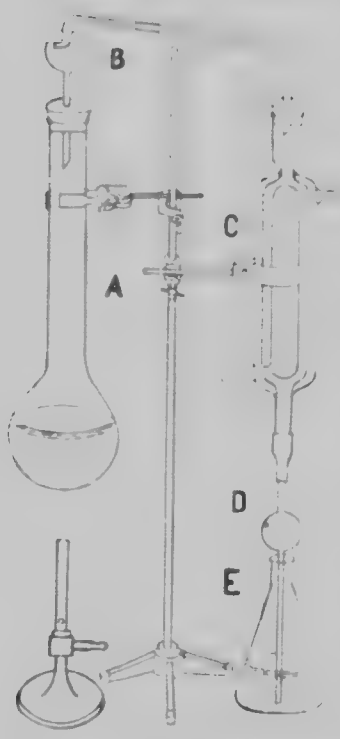
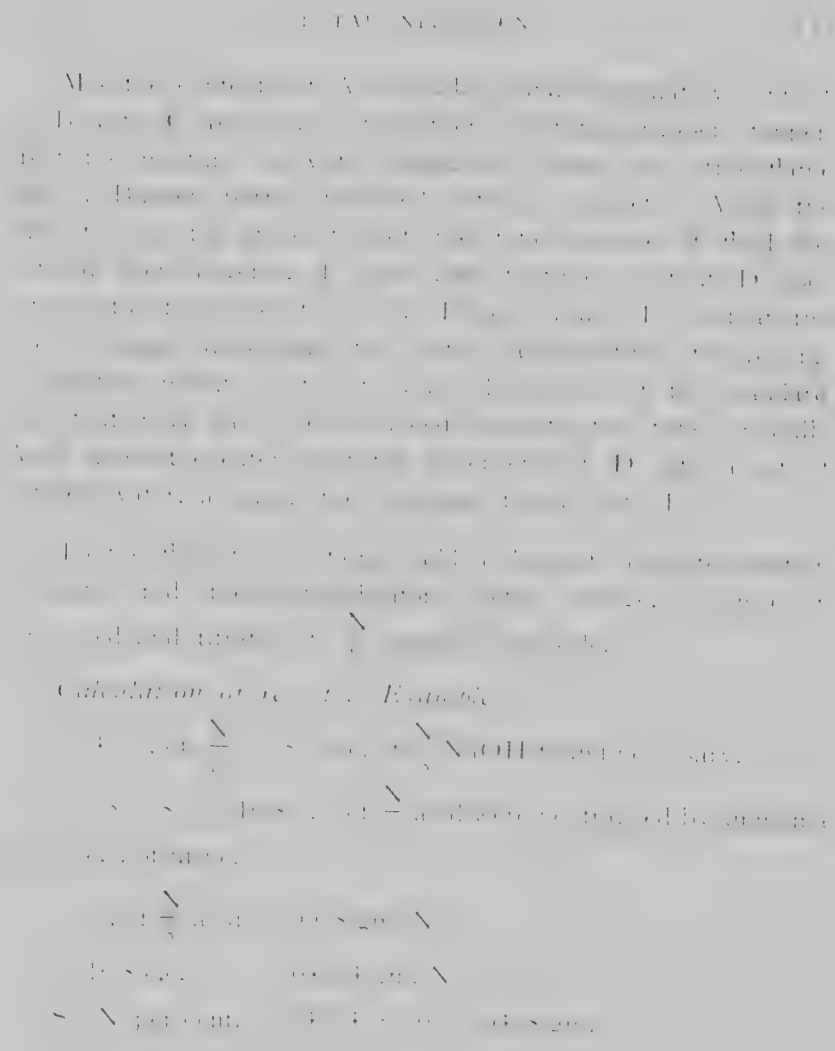


Fig. 7. Apparatus [illegible]
Met[illegible]

[illegible] nat the lower [illegible] D [illegible] nd to E. [illegible] E being sucked back by a [illegible] stillation. D [illegible] Davies', [illegible]

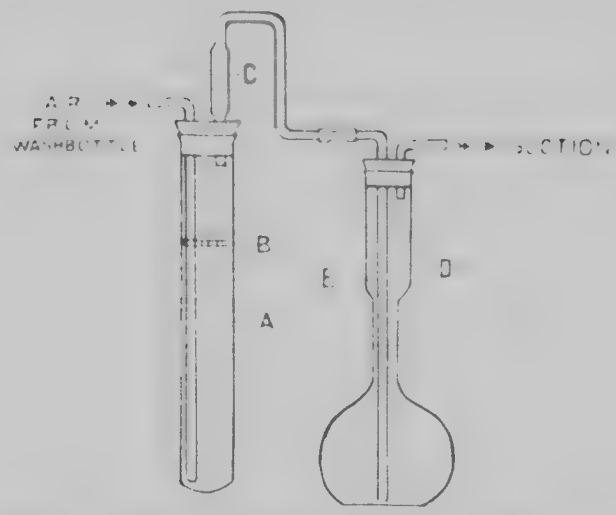
[illegible] the flask A [illegible] sodium hydroxide, burn it down [illegible] as to form otton layer; [illegible] prevented.

Fit the glass tube B [illegible] ell-fitting rubber stopper. [illegible] prevent any of the alkaline fluid [illegible] distillate.



507. The estimation of total nitrogen by Folin's micro-chemical method.

Principle. A small volume of urine is de[illegible] by [illegible] acid as in Kjeldahl's method. The ammonia is drawn over [illegible] acid and the solution treated with Nessler's reagent. The [illegible] ment of ammonia is determined colorimetrically by comparison with a standard solution of ammonium sulphate simultaneously Nesslerised.


C
B
A
D

[illegible]. Add [illegible] c.c. of pure sulphuric acid, 1 gram of [illegible] 1 drop of 5 per cent. [illegible] [illegible] present purpose. [illegible] [illegible] [illegible] and [illegible] c.c. of water, [illegible] [illegible] to prevent [illegible].

Distillation of the Ammonia. [illegible] apparatus [illegible] shown in Fig. 8. Transfer [illegible] side [illegible] the mixture in the test tube A, and [illegible] the ammonia into [illegible] c.c. of $\frac{N}{10}$ hydrochloric acid [illegible] distilled water [illegible] the [illegible] flask D. The [illegible] may be moderate for the first two minutes [illegible] for the next eight minutes it should be as rapid as possible. Disconnect and dilute the contents to about [illegible] c.c., washing the tube E with [illegible] water added.

Preparation of the Nesslerised solutions. In another 100 c.c. measuring flask place 5 c.c. of standard ammonium sulphate solution, containing 1 mgrm. of nitrogen, and dilute to about [illegible] c.c. To each flask add 5 c.c. of cold saturated solution of Rochelle salt to prevent the formation of a cloud on adding Nessler's solution. Nesslerise both solutions as nearly as possible at the same time with 5 c.c. of Nessler's reagent diluted *immediately* beforehand with 25 c.c. of water. Fill both flasks to the mark with water and mix.

Determination of the depth of colour. This is done by means of a Duboscq colorimeter (See Fig. 54, p. 192). In one of the chambers B place some of the unknown solution, in the other some of the standard ammonia solution. Place the tube D of the standard at a certain depth (20 mm. is usually the best) and adjust the other tube until the colours match. Several readings should be taken, moving the unknown from below and from above.

Calculation of result. Example:

$$\frac{\text{Height of standard}}{\text{Height of unknown}} = \frac{\text{mm.}}{\text{mm.}}$$

Solution I c.c. diluted to contains 0·04 mgm. nitrogen.

Urine was diluted 1 in 1.

So 10 c.c. of urine contains 0·4 mgm. nitrogen.

Preparation of the standard solution of ammonium sulphate. Pure ammonium sulphate is decomposed by means of caustic soda and the ammonia passed into pure sulphuric acid by means of the air current. When all the acid has been neutralised, the solution is partially evaporated and the salt precipitated by alcohol. It is redissolved in water, reprecipitated by alcohol, and dried in a desiccator over sulphuric acid.

9·4585 gm. of the ammonium sulphate are dissolved in water and the volume made up to 1 litre. (Stock solution).

100 c.c. of the stock solution are diluted to form 1 litre (standard solution).

5 c.c. of the standard solution contain 1 mg. nitrogen.

Preparation of Nessler's reagent.

Dissolve 62·5 gm. of potassium iodide in about 250 c.c. of distilled water, set aside a few c.c. and add gradually to the larger part a cold saturated solution of mercuric chloride (of which about 500 c.c. will be required) until a faint permanent precipitate is produced. Add the reserve portion of the potassium iodide and then mercuric chloride very gradually till a slight permanent precipitate is again formed.

Dissolve 150 gm. of solid potassium hydroxide in 150 c.c. of distilled water, allow the solution to cool and add it gradually to the above solution and make the volume up to 1 litre. Allow to settle, decant the clear liquid into another bottle and keep in the dark. The reagent improves on keeping.

The estimation of ammonia by Folin's method.

Fig. 9. Folin's apparatus for estimating ammonia

A. Wash bottle containing acid.
B. Tall acrometer cylinder containing urine.
C. Bottle containing standard acid.
D. Calcium chloride tube, loosely packed with cotton wool, to prevent any sodium carbonate being carried over into C.
E. Folin's absorption tube, to bring the air into intimate contact with the acid.

Use the apparatus shown in Fig. 9*.

Into C measure 20 c.c. of $\frac{N}{10}$ sulphuric acid and two drops of a dilute solution of methyl red, or Alizarin red.

Into B measure 25 c.c. of urine, add 10 c.c. of kerosene oil (to prevent foaming) and one gram of anhydrous sodium carbonate. Connect up the apparatus at once, and draw air through for two hours.

* The parts of the apparatus can be obtained from Messrs. J. Griffin and Sons or Messrs. Baird and Tatlock.

206. The estimation of ammonia by Folin's microchemical method.

The estimation of ammonia by the formaldehyde method.

Principle. [illegible]

[illegible]

Preparation of formaldehyde solution. [illegible]

Method of estimation. [illegible]

Calculation of results. [illegible]

II. The estimation of urea by Benedict's method.

Principle. Urine is treated with [illegible] and heated to 165° C. for one hour. The urea is thus hydrolysed to ammonium compounds which are retained by the acid mixture. The fluid is diluted, made alkaline with sodium carbonate, and the ammonia distilled into standard acid. The

amount of this neutralised by the ammonia formed is determined by titration with standard alkali. The ammonia nitrogen of the urine must be previously determined.

Method. 5 c.c. of urine are measured into a wide Jena test-tube (200 × 30 mm.) and treated with about 3 grams of potassium bisulphate and 1 to 2 grams of zinc sulphate. A little powdered pumice and a few drops of paraffin are introduced from time to time during and spattering must be avoided. The mixture is boiled practically to dryness, either over a naked flame or, more conveniently, by floating the tube in a bath of sulphuric acid kept at about 130° C. A convenient bath is a tall narrow Jena glass, or, preferably, porcelain beaker of about 800 1,000 c.c. capacity, two-thirds filled of sulphuric acid.

The tube must be immersed for at least three-fourths of its length in the sulphuric bath. This can be done by clamping the tube to the edge of the bath. Raise the temperature of the bath to 162 165° C. and maintain there for one hour. Remove the tube and allow it to cool somewhat. Wash off the acid mixture from the top. Wash the contents by means of hot water quantatively into a 500 c.c. Jena flask (A, fig. 7, p. 120). The volume of the fluid in the flask should be about 300 c.c.

Fig. 1 p. 19 the apparatus arranged for Kjeldahl's method, placing 25 c.c. of $\frac{N}{5}$ sulphuric acid in D. To A add about 25 c.c. of a saturated solution of sodium carbonate. Connect up the apparatus and distil for about forty minutes, till about one-half of the fluid has passed over. Boil the fluid in D to remove excess of CO_2, cool and titrate with N/10 sodium hydroxide, using methyl red, cochineal or methyl orange as an indicator.

Calculation of results. Example:

Ammonia nitrogen of 5 c.c. urine was previously found to correspond to 10 c.c. $\frac{N}{10}H_2SO_4$ = 5 c.c. $\frac{N}{5}H_2SO_4$.

So ammonia nitrogen of 5 c.c. urine = 1 c.c. $\frac{N}{5}H_2SO_4$.

In this exercise [illegible] of $\frac{N}{10}$ NaOH, [illegible] of $\frac{N}{5}$ NaOH [illegible] the [illegible] $\frac{N}{5}$ H_2SO_4.

[illegible] amount of $\frac{N}{5}$ H_2SO_4 [illegible] [illegible]

[illegible]

[illegible] $\frac{N}{5}$ H_2SO_4 [illegible] N [illegible]

[illegible]

[illegible]

[illegible]

The estimation of urea by Folin's microchemical method.

Principle. [illegible] with [illegible] and [illegible] and heated. [illegible] at 100° C. and at this temperature the urea is readily [illegible] and ammonia, which is retained [illegible] acetate. Solid potassium [illegible] is added, and the ammonia [illegible] and [illegible]. The solution is Nesslerised and the colour compared with that of a standard solution of ammonium sulphate [illegible] and simultaneously Nesslerised.

Method. The urine must be diluted so that [illegible] contains 0·5 to 1·0 mgrms. of urea nitrogen. Usually [illegible] is about correct. Into a large *dry* Jena test tube [illegible] of dry potassium acetate, [illegible] of 50 per cent. acetic acid, a small fragment of granulated zinc to prevent bumping and a temperature indicator (see below). To the tube add [illegible] of the diluted urine by means of an accurate pipette. The test tube is then closed by means of a rubber stopper carrying an empty narrow "calcium chloride tube," with [illegible] of 0·25 mm. bore. The test tube is held by a clamp, so that it can be readily raised or lowered. Heat is applied by means of a [illegible] adjusted

shielded from air currents by means of a chimney or a battle-ess beaker. The flame should be about 6 cm. long.

The acetate dissolves and the mixture begins to boil. The temperature indicator should show that the temperature has reached 153° C. to 160° C. Heating is continued for ten minutes after the temperature has been attained. The temperature must not reach 165° C., at which point the acetate cakes and solidifies. Remove the apparatus from the flame and dilute the contents with 50 c.c. of water, adding it by means of a pipette through the calcium chloride tube so as to rinse the sides of the tube and the bottom of the rubber stopper from traces of ammonium acetate which may be there; add 100 c.c. of saturated sodium hydroxide solution and aspirate the ammonia into acid exactly as described on p. 179. Estimate the nitrogen in the nitrometer against Pregl's standard gas as described above.

Temperature indicator. This consists of powdered cadmium iodide of mercury ($CdHgI_4$) sealed in fusion tubes 10 to 15 mm. in length and 1 to 2 mm. in diameter. The salt is bright red at ordinary temperatures. It turns lemon yellow at 115° C. and melts to a clear dark red liquid at 159° C. The same indicator cannot be used twice within 24 hours.

The salt is prepared by heating in a dry state intimately mixed mercuric chloride (0·7 gm.) and mercuric iodide (1·45 gm.) in molecular proportions for six to eight hours at 150° to 160° C. At the end of the heating the product should be powdered and kept dry in a sealed up as indicated.*

Calculation of results. Example.

Uncalculated Dumas.

Height of standard	190 mm.
Height of nitrometer	18 mm.

Sodium acetate solution + Dumas N as urea and ammonia.

* [illegible] Merck's Report, 1911, p. 1. Merck, Darmstadt.

So 100 c.c. urine contain $1000 \times 1{\cdot}11$ mgm. $= 1{\cdot}11$ gm. of urea and ammonia-N.

Ammonia-N was found to be 0·45 gm. per 100 c.c.

So urea-N per 100 c.c. $= 1{\cdot}11 - 0{\cdot}045 = 1{\cdot}065$ gm.

$$\text{Urea} = 1{\cdot}065 \times \frac{CO(NH_2)_2}{N_2} = 1{\cdot}065 \times \frac{60}{28} = 2{\cdot}28 \text{ per cent.}$$

NOTES.—1. The method has to be modified for diabetic urine, owing to the [illegible]

The urine is diluted 100 times and 1 c.c. of the diluted urine decomposed [illegible]

The ammonia is driven into another tube containing about 2 c.c. of water and 0·5 c.c. of $\frac{N}{10}$ HCl. To this tube are added first 2 c.c. of water, and then [illegible] c.c. of 1 in 5 Nessler's solution. The coloured solution is washed into a 10 c.c. [illegible] flask and the volume made up to 10 c.c. The colour is determined against that of the usual standard containing 1 mgm. of nitrogen per 100 c.c. of solution.

313. The estimation of urea by the hypobromite method.

Remarks. This is the standard method for the clinical estimation of urea. It is of the utmost importance for the student to realise that the method is essentially inaccurate and may lead to very erroneous conclusions. The nitrogen evolved comes from urea, ammonia, and to a small and undetermined extent from creatinine and other nitrogenous constituents. Further, urea does not evolve the whole of its nitrogen in the form of gas, so that allowances have to be made. Since the proportion evolved varies with differences in the composition of the fluid it is obvious that no certain deductions can follow such a determination. It is with the utmost diffidence that the method is given. It is most certainly not to be recommended.

Principle. Urine is treated with an alkaline solution of sodium hypobromite and the amount of urea calculated from the volume of nitrogen evolved.

The reaction that takes place is as follows:

$$CO(NH_2)_2 + 3NaBrO + 2\ NaOH = N_2 + 3\ NaBr + Na_2CO_3 + 3\ H_2O.$$

Hence 0·1 grams urea evolve 25 grams N. = 11·2 litres, and 1 gram urea evolves 373 c.c. N.

Practically urea and uric acid only are decomposed, the other 4·4 per cent. of the nitrogen being converted into nitrates, ammonia, etc.

Apparatus. See fig. 54. A is a tall cylinder filled by a compound tube with water *b*. The water enters the burette as soon as the stopper *c* is opened, which is provided with a bulb. The gas passes through the T piece and rubber tube *d* into the measuring tube *e*. The side tube of the T piece is connected by about two feet of small rubber tubing to a glass tube *a* passing through a rubber stopper into a wide mouthed bottle of about 60 c.c. capacity. This bottle is placed in a pail of water, supported at such a height that the burette can be lifted nearly out of the tall cylinder without stretching the rubber connections. A small glass bottle or short tube of 10 to 15 c.c. capacity is also required (*d*).

Fig. 54. Apparatus for determination of urea by hypobromite method.

For the method of preparing the hypobromite solution see Ex. 149.

Method of Analysis. Place 25 c.c. of freshly prepared hypobromite solution in (*e*). Put 2 c.c. of urine, accurately measured, in the small bottle (*d*), and place this inside the other by means of a pair of forceps, taking great care not to upset any urine into the hypobro[illegible] Fit the

rubber cork tightly into the bottle and place it over the tube. See that the bottle is as low as possible, that the whole bulb is immersed in water, that no trace of air-bubble remains in the bottle, and that the tube is open. Let the apparatus stand about a minute to enable the temperature of the water in the bottle to assume such a position that the water in the tube is neither rising nor falling, and then close the tube under the water by the pinch-cock. Note down the position of the water in the tube, remove the bottle from the water and shake the contents of the bottle thoroughly.

Gently suck or press some of the liquid from the bottle into the tube to prevent the water from being sucked back into the bottle. Then let the liquid run back and repeat the process until no more gas bubbles are seen to rise when the liquids are mixed. Place the bottle back in the water for about 5 minutes to cool. Raise the tube so as to fill the tube with water to the same level as in the bottle, the two being then under atmospheric pressure. Read the level of the mercury as before. The difference in the two readings gives the volume of nitrogen evolved. Ascertain the temperature of the water and the barometric pressure.

Calculation of results.

Let the temperature be t C., the tension of aqueous vapour at this temperature be T mm. (See Appendix) and the barometric pressure be B mm. of mercury. Let v c.c. of nitrogen be measured under these conditions. At 0 C. and 760 mm. this will become

$$v \times \frac{273}{273 + t} \times \frac{B - T}{760}$$

N c.c. 357 c.c. of N are evolved from 1 gram of urea.

v' c.c. are evolved from $\frac{v'}{357}$ grams of urea.

x c.c. of urine contain $\frac{x'}{357}$ gram urea.

and 100 c.c. of urine contain $\frac{100x'}{357}$ gram urea.

[illegible]

[illegible]

317. The estimation of uric acid by the Folin-Schaffer method.

Principle. The mucoids and some of the phosphates are precipitated by ammonium sulphate containing uranium acetate and acetic acid. The filtrate is rendered alkaline by ammoniac. Ammonium urate separates out. This is washed free from chlorides with ammonium sulphate, suspended in water and titrated with $\frac{N}{20}$ potassium permanganate.

Preparation of Solutions.

1. Uranium acetate solution. Dissolve 500 gm. ammonium sulphate, 5 gm. uranium acetate and 60 c.c. of 10 per cent. acetic acid in 650 c.c. of water. The volume of the solution is almost exactly 1000 c.c.

2. $\frac{N}{20}$ potassium permanganate. Dissolve 1·581 gm. of the pure salt in distilled water and make the volume up to 1000 c.c.

Method. 200 c.c. of urine are treated with 50 c.c. of the Folin-Schaffer reagent, allowed to stand for 20 minutes and filtered through a dry paper into a dry flask.

Measure 125 c.c. of this filtrate into a beaker, previously marked at the 100 c.c. level by means of a label, add 5 c.c. of concentrated ammonia, and allow it to stand for 24 hours. Carefully filter off the supernatant fluid through a hardened filter paper, and wash the precipitate on to the paper with 10 per cent. ammonium sulphate. Wash the precipitate twice more with this reagent to remove the chlorides.

Remove the paper from the funnel, open it, and by a fine jet of hot water, rinse the precipitate back into the beaker. Cool under the tap and make the volume up to 100 c.c. with distilled

water. Add 15 c.c. of concentrated sulphuric acid and titrate at once, without cooling, with $\frac{N}{20}$ potassium permanganate from a burette, which must have a glass tap.

During the titration the fluid in the flask must be kept in [illegible] movement. Each drop of the permanganate is at first decoloured almost immediately, before it has had time to diffuse through the liquid and react [illegible]. The first instantaneous appearance of a [illegible] throughout the whole body of the solution marks the end point of the titration. The colour disappears very rapidly, but it will now be found that if another drop of permanganate be added, it has time to diffuse through the fluid, [illegible].

Calculation of the result.

1 c.c. of the [illegible] = [illegible] gm.

1 c.c. of $\frac{N}{20}$ permanganate = 0·00375 gm. uric acid.

Add to the result 0·003 gm. for the 100 c.c. to allow for the solubility of ammonium urate in the reagents.

Example.

[illegible]

12·4 c.c. of permanganate required.

Percentage [illegible] = 12·4 × 3·75 + [illegible]

= [illegible] + [illegible].

= [illegible].

Total in [illegible] = [illegible] + [illegible] = 0·79 gm.

Uric acid in urine = 0·79 × [illegible] = 1·5 per cent.

315. The estimation of uric acid by Folin's microchemical method.

Principle. Urine is evaporated to dryness and extracted with ether and alcohol to remove polyphenols. The residue is dissolved in dilute alkali and treated with Folin's uric acid reagent. The fluid becomes coloured blue and is compared colorimetrically with a standard solution of uric acid similarly treated.

Let the depth [illegible] when on a parallel [illegible]

Suppose [illegible]

Then [illegible]

So [illegible]

31. The estimation of creatinine by Folin's method.

Principle. [illegible] with picric acid [illegible] the colour [illegible] with a standard [illegible] of potassium dichromate [illegible]

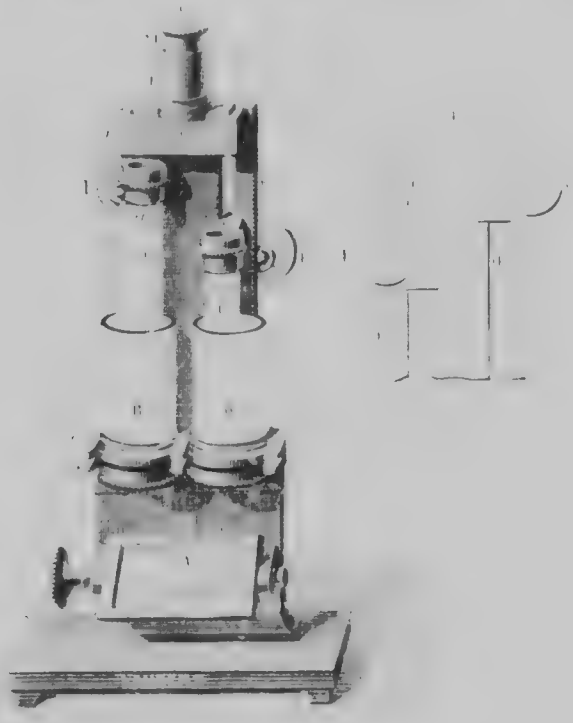


Fig. 11. [illegible]
[illegible]

Autenrieth's method. A colorimeter (Duboscq) is the most [illegible] ... is the com- [illegible] ... The tubes D are [illegible] at the [illegible]

[illegible]

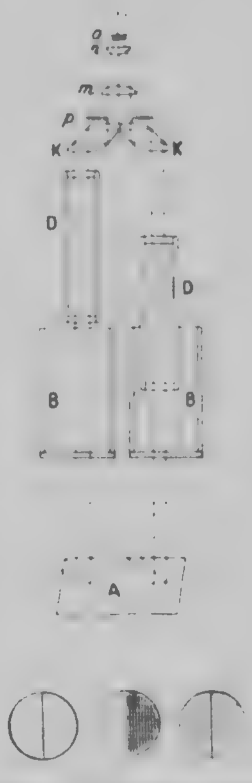


FIG. [illegible] [illegible] Duboscq [illegible]

[illegible] ... the two tints match. The [illegible] ... Several determinations should be made, moving the second tube both [illegible] and below. The readings [illegible]

Preparation of the dichromate solution. Dissolve 1·5[illegible] gm. of pure [illegible] dichromate in water and make the volume up to 1000 c.c.

Method of analysis. Measure 10 c.c. of the urine with a pipette into a 500 c.c. [illegible] flask. Add 15 c.c. of saturated picric acid in water (about 1·[illegible] per cent.) and 5 c.c. of 10 per cent. caustic soda. Mix and allow to stand for 5 minutes. Fill the

[illegible] the standard solution. Place the deproteinized [illegible] Place the [illegible] romate tube at a depth of 8 mm. and determine the position of [illegible]

Calculation of result.

[illegible]

If the depth of the layer be x mm., then the creatinine in [illegible]

Note. If the reading be less than 5 mm., the urine must be carefully [illegible]

317. **The estimation of the titration acidity by Folin's method.** Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask, add 1·5 gm. of finely powdered neutral potassium oxalate, 3 drops of 1 per cent. phenolphthalein and shake the mixture vigorously for 2 to 3 minutes. Titrate with $\frac{N}{10}$ sodium hydroxide, until a permanent faint pink colour is produced.

Calculation. Express the result in terms of $\frac{N}{10}$ soda. Thus if 7 c.c. of soda are required for 25 c.c. of urine, the acidity of the urine is equivalent to 28 c.c. of $\frac{N}{10}$ sodium hydroxide per 100 c.c.

Note. [illegible] Each individual worker should [illegible]

2 For the estimation of the true acidity (the concentration of the hydrogen [illegible]

The estimation of chlorides by Volhard's method.

Principle. The chlorides are precipitated from urine by a [illegible] solution, a ferric salt being used as an indicator [illegible] nitrate.

Reagents required.

(i) Standard silver nitrate [illegible] 29·075 grams of pure fused silver nitrate in distilled water and making up accurately to one litre. The solution should be kept in the dark.

1 c.c. corresponds to ·01 gram NaCl (·00606 gram Cl).

(ii) Solution of potassium sulphocyanide made by dissolving 8 grams of the salt in a litre of distilled water.

(iii) Pure nitric acid, quite free from chlorine.

(iv) A concentrated solution of iron alum.

Standardisation of the Sulphocyanide. In a beaker place 10 c.c. of the silver nitrate, accurately measured: add 5 c.c. of pure nitric acid, 5 c.c. of iron alum and 80 c.c of distilled water. Titrate the whole with the sulphocyanide from a burette until a faint permanent red tinge is obtained. Note the amount required for the 10 c.c. of silver nitrate.

Method of Analysis. In a 100 c.c. cylinder or measuring flask place 10 c.c. of urine, accurately measured by a pipette, [illegible] c.c of the standard silver solution, also accurately measured, about 4 c.c. of pure nitric acid, and 5 c.c. of the iron alum. Add distilled water till the 100 c.c. mark is just reached, and mix thoroughly by

Calculation of results.

Standardisation of sulphocyanide shows that

KCNS = x c.c. standard silver.

s c.c. KCNS.

S c.c. KCNS

S c.c. KCNS = S × x c.c. of standard silver.

We added 20 c.c. standard silver to 10 c.c. urine.

standard silver = ·01 gram NaCl.

S × x × ·01 gram NaCl

Example.

KCNS = c.c. AgNO$_3$.

S c.c. KCNS = $\frac{\text{S}}{\text{x}}$ c.c. AgNO$_3$ = x.

50 c.c. urinary filtrate required 11·6 c.c. KCNS = S.

100 c.c. " " " 23·2 c.c. 2 S.

23·2 c.c. KCNS = $\frac{23 \cdot 2 \times 1 \cdot 3}{1 \cdot 0}$ = 11·8 c.c. AgNO$_3$.

So 20 − 11·8 = 8·2 c.c. AgNO$_3$ = NaCl in 10 c.c. urine.

NaCl in 10 c.c. is 8·2 × ·01 = ·082.

NaCl in 100 c.c. is 0·82 gram.

319. The estimation of phosphates.

Principle. Urine is heated to boiling point, and titrated whilst hot with a standard solution of uranium acetate, which gives a precipitate of $(UO_2)HPO_4$ with phosphates in acetic acid solution.

Reagents required.

(1) A standard solution of potassium permanganate, containing 0·395 gram of the salt per litre of solution. This solution is best prepared by dissolving 4 grams of the salt in 10 litres of water.

(2) Crystallised sodium oxalate, prepared by recrystallising the commercial salt from water, and drying at 100° C. for some hours.

(3) A standard solution of sodium oxalate, containing 67 grams of the salt per litre of solution. This solution is prepared by dissolving 67 grams of the salt in water, and making up to 1 litre. When required for use, 10 c.c. of the solution are diluted with water to 100 c.c. and heated to 60° C. About 10 c.c. of dilute sulphuric acid (1 vol. of acid to 3 vols. of water) are added to the hot solution, and the solution is titrated with the permanganate solution prepared as directed in (1). The titration is repeated until two consecutive readings agree. The number of c.c. of permanganate solution required to oxidise the 10 c.c. of sodium oxalate solution is then determined. Let this number be n. Then the number of c.c. of permanganate solution required to oxidise 1 c.c. of the sodium oxalate solution is $\frac{n}{10}$. Let this number be m. Then $\frac{1}{0{\cdot}0134m}$ is the number of c.c. of such a standard solution of sodium oxalate which will be required to oxidise 1 c.c. of the permanganate solution.

(4) Standard solution of uranium acetate. Dissolve 35 grams of uranium acetate in 1 litre of water. Make the solution exactly decinormal. Standardise the solution by titrating 10 c.c. of it with the standard solution of sodium oxalate, using a drop of dilute phosphate of sodium on a porcelain tile as indicator. Repeat the titration a few times, and take the mean of the readings. Let this mean be p c.c. Then $\frac{p}{10}$ is the number of c.c. of the standard solution of sodium oxalate which will be required to oxidise 1 c.c. of the uranium acetate solution. Let this number be q. Then $\frac{1}{0{\cdot}0134q}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of the permanganate solution. Let this number be r. Then $\frac{r}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 gram of urea. Let this number be s. Then $\frac{s}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of urine containing 1 gram of urea. Let this number be t. Then $\frac{t}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of urine containing 1 per cent. of urea. Let this number be u. Then $\frac{u}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of urine containing 1 per cent. of urea after treatment with bone-black. Let this number be v.

(5) A standard solution of sodium phosphate. Dissolve 50 grams of the crystallised salt in water, and make up to 1 litre. Add to the solution enough of a solution of uranium acetate to produce a precipitate with a drop of the solution on a porcelain tile. Filter off the precipitate, and evaporate the filtrate to dryness on the water-bath. Weigh out 0·5 gram of the dry residue, and dissolve in water. Add 10 c.c. of dilute sulphuric acid, and titrate with the standard solution of uranium acetate. Let the number of c.c. of uranium acetate solution required be w. Then $\frac{w}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of the sodium phosphate solution. Let this number be x. Then $\frac{x}{0{\cdot}0134}$ is the number of c.c. of the uranium acetate solution which will be required to oxidise 1 c.c. of urine containing 1 per cent. of urea after treatment with bone-black and sodium phosphate. Let this number be y.

Method of Analysis. [illegible]

Calculation of results.

[illegible]

[illegible]

The estimation of total sulphates by Folin's method.

[illegible]

Calculation. Weight of $BaSO_4$ × [illegible] = SO_4 present.

Notes. [illegible]

The estimation of inorganic sulphates by Folin's method.

Place 25 c.c. of urine and [illegible] c.c. of water [illegible]. Add 10 c.c. [illegible] and 1 [illegible] of concentrated HCl [illegible] water. Add [illegible]

[illegible] in the proportion [illegible] and proceed as directed.

Calculation. [illegible]

Ethereal Sulphates.

[illegible] sulphates [illegible]

The estimation of total sulphur by Benedict's method.

[illegible]

The estimation of albumin by Esbach's method.

[illegible]

Calculation. [illegible]

[illegible] Esbach's

64. The estimation of albumin by Scherer's method.

Measure 50 c.c. of urine into a beaker. Place it in a water bath and raise the temperature to 60° C. Add 1 per cent. acetic acid, drop by drop, to obtain a complete separation of the protein matter. It is better to add an excess. Raise the temperature to boiling and keep it so for a few minutes. Filter the mixture through paper that has previously been washed and weighed. Wash the precipitate on the filter with hot water, 95 per cent. alcohol and ether. Dry the paper and its contents in a water oven at 110° C. till the weight is constant. [illegible] described in the first chapter of this paper.

CHAPTER XI.

DETECTION OF SUBSTANCES OF PHYSIOLOGICAL INTEREST.

A. Fluids.

1. Neutralise a drop of the fluid on a piece of red litmus paper with dilute ammonia, or sodium carbonate solution, and then render distinctly acid with acetic acid. Divide the neutralised fluid into two portions. To one portion add a few drops of ferric chloride solution. If a red colour appears, acetoacetic acid is present. Boil the other portion for a few minutes, cool, and add ferric chloride solution. If a red colour appears, acetoacetic acid is present. Boiling should not be continued for more than a few minutes, as acetoacetic acid is decomposed on prolonged boiling.

2. Note any characteristic smell of urine, bile, etc.

3. Note the colour and appearance of the fluid: opalescence suggests starch, glycogen, or certain protein solutions; coloured fluids suggest bile, blood or urine.

4. Note the reaction to litmus. An acid reaction excludes the presence of semen, nucleoproteins, caseinogen, and usually, earthy phosphates.

5. If acid to litmus test for free HCl by Günzburg's test. (Ex. 187A.)

6. Sprinkle some flowers of sulphur on the surface of a portion of the fluid in a test tube. If the particles fall through the surface, bile salts are present. (Ex. 43.) Confirm by Pettenkofer's test. (Ex. 45.)

7. If the fluid be brown or green, apply Gmelin's test (Ex. 175) for bile pigments.

8. If the fluid be red or brown, examine for blood; pigment or derivatives by Table I.

9. If there are any reactions for proteins, the presence of ferments, examine by Table G. If no proteins or reactions for proteins are obtained, ferments are probably absent.

10. Examine for proteins by Millon and the biuret reactions (Ex. 2 and 4). If they are present, proceed as directed in Table A, B or C, according to the reaction or quantity.

11. If proteins are absent, proceed to Table F.

12. Test for uric acid: the fluid is allowed to stand for twenty-four hours in a warm place. A deposit will usually appear, which is uric acid or a urate. Uric acid may separate out at once on adding hydrochloric acid; uric acid may separate out at once on adding water. Make another portion of the solution alkaline with ammonia, saturate with NH_4Cl, and apply the murexide test to the precipitate thus obtained. (Ex. 21.)

13. If the fluid be alkaline, treat a little with a solution of calcium chloride. A white curdy precipitate indicates the presence of soaps. (Their presence should be confirmed by the methods given in Ex. 128.)

Table A.

Analysis of an **acid** solution containing protein.

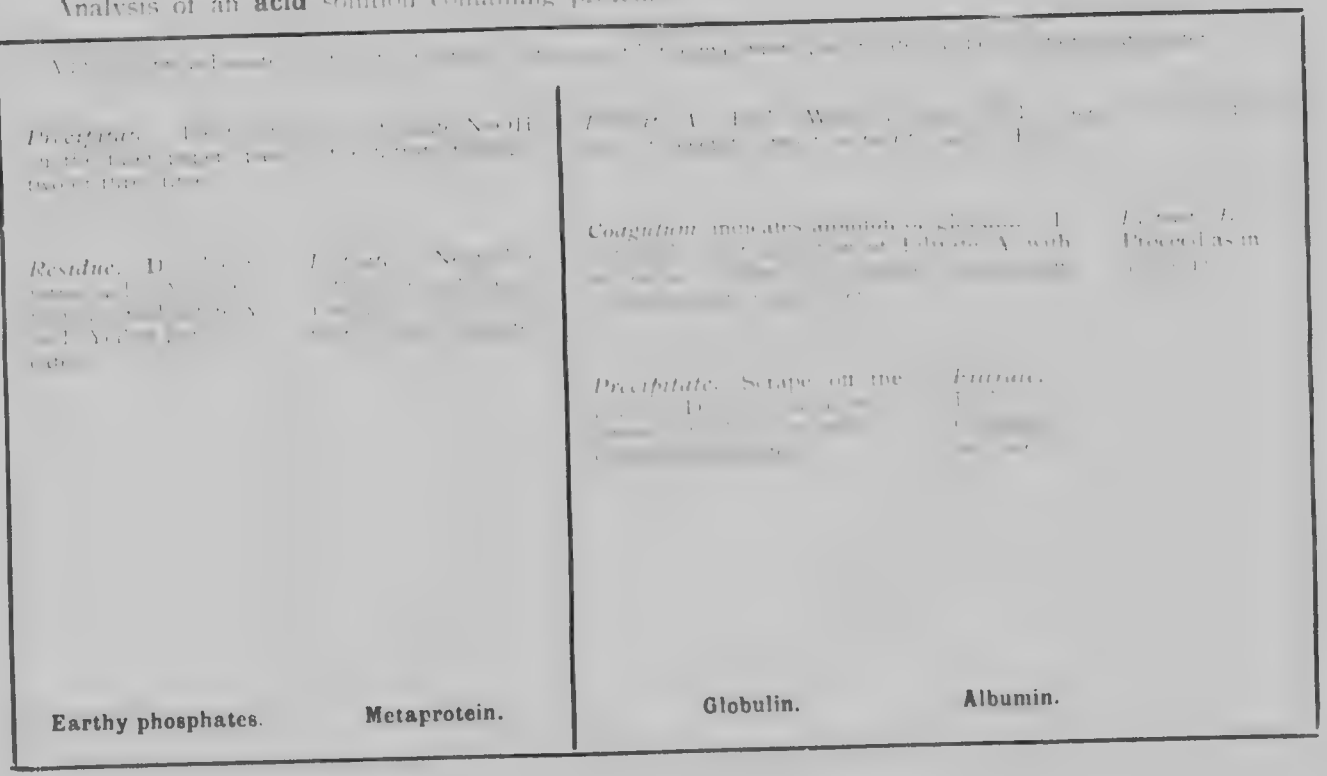


Table B.

Analysis of a **neutral** solution containing proteins.

Precipitate (i) **Bile salts with any protein.**

Mucin.

(iii) **Casein, caseinogen or nucleoprotein.**

Table C.

Analysis of an **alkaline** solution containing proteins.

Precipitate (i) **Bile salts with any protein.**

(ii) **Fatty acids.** Test original solution for soaps by Ex. 128

(iii) **Mucin.** Insoluble in strong acetic acid.

(iv) **Casein, caseinogen or nucleoprotein.**

Contain phosphorus. (See Ex. 21.)

Ppt. Soluble in excess of

Metaprotein

Table D.

albumoses, peptones gelatin.

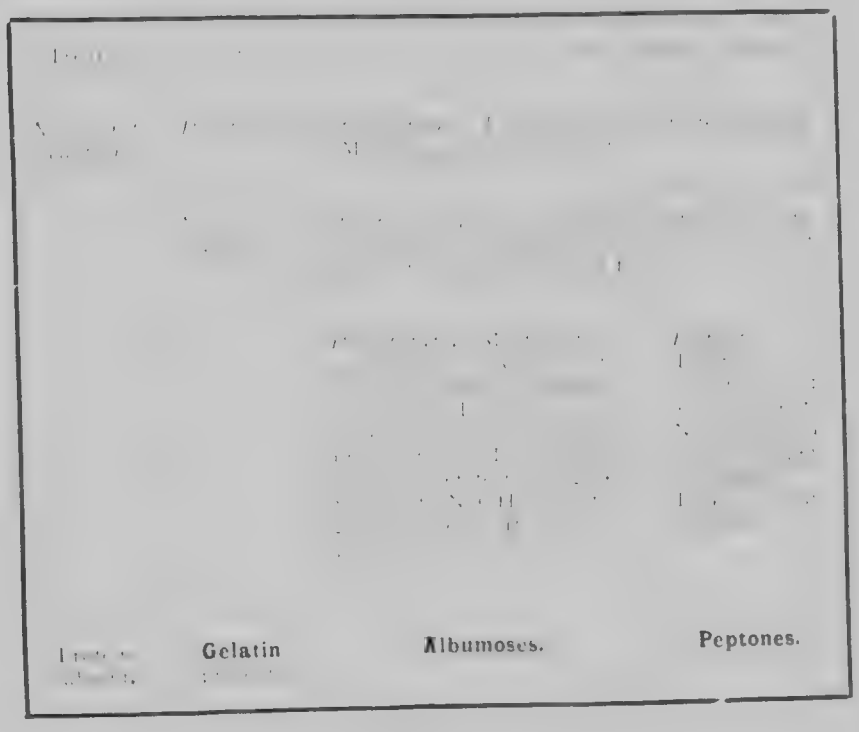


Table E.

carbohydrates urea.

glycogen

starch.

erythro-dextrin.

reducing sugars.

cane-sugar

urea.

Table F.

Table G.

Examination for **ferments.**

Take two reactions [illegible]

I. Marked acid.

Examine for pepsin. [illegible]

Neutralize [illegible] examine for pepsin. (See Ex. [illegible])

II. Feeble acid [illegible]

Examine for pepsin. [illegible]

Examine for pepsin. (See Ex. [illegible])

Examine for rennin. [illegible]

Examine for trypsin. [illegible]

Examine for ptyalin. [illegible]

III. Distinctly alkaline.

Examine for ptyalin. [illegible]

Examine for trypsin. (See Ex. [illegible])

Examine for pepsin. (See Ex. [illegible])

Perform control experiments in all cases. (See Ex. [illegible])

A few special hints on the examination of physiological fluids.

1. It is impossible to obtain a test reaction of albumin or globulin in an acid or alkaline fluid. The reaction must be *neutral* or only very faintly acid.

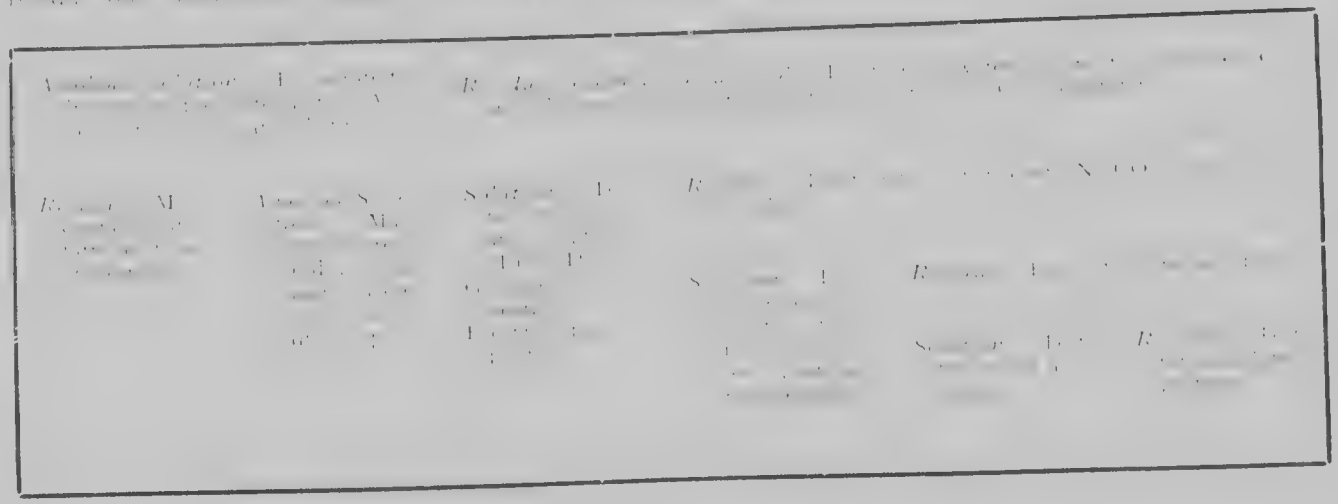
2. A little litmus solution in the fluid does no harm, and often reminds one that the reaction changes after boiling (owing to the evolution of CO_2).

3. In testing for peptones, after removing the albumoses by saturation with ammonium sulphate, the biuret test succeeds only if at least two volumes of 40 per cent. soda are used. The test will not be obtained with the ordinary 5 per cent. soda.

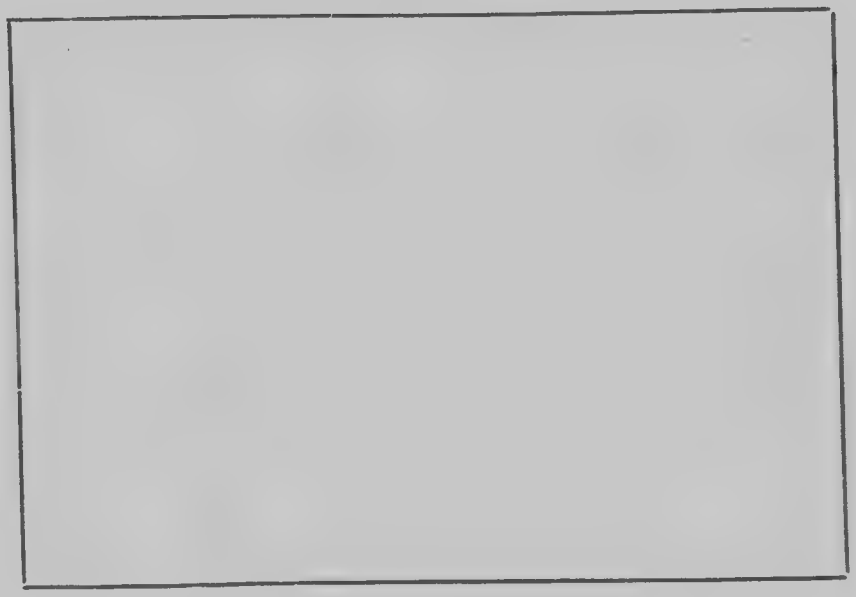
4. Gelatin reacts very much like the albumoses, except that it does not yield the glyoxylic reaction.

B. Solids.

Analysis of a Solid for substances of Physiological Interest.



APPENDIX.



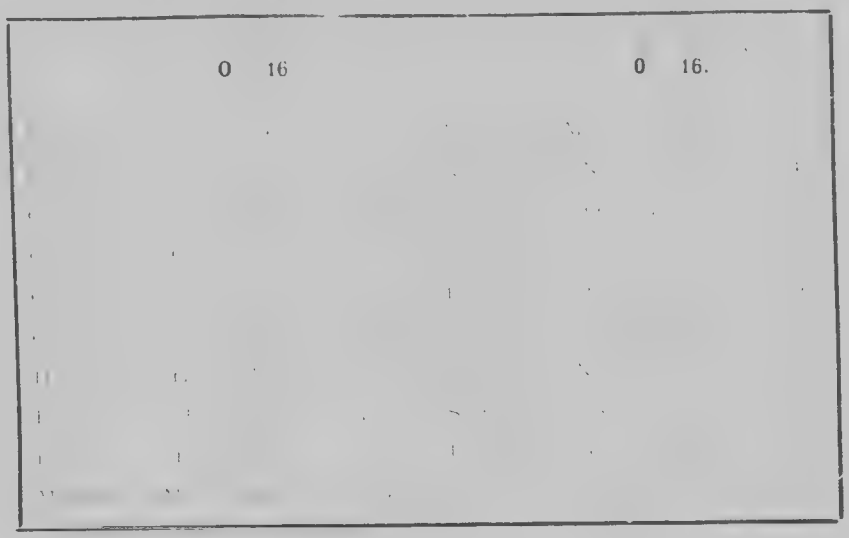

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CHART FOR RECORDING THE ABSORPTION SPECTRA
OF PIGMENT SOLUTIONS

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